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Alterations in the Structure of Denervated Mammalian Skeletal Muscle with particular referance to the Sarcolemma

by

© Ulka R. Tipnis

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Alterations in the Structure of Denervated Mammalian Skeletal Muscle with particular reference to the Sarcolemma" submitted by Ulka R. Tipnis in partial fulfilment of the requirements for the degree of Master of Science, in Cell Biology.

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Date March 28th 1977

ABSTRACT

Synaptic and non-synaptic regions of innervated and denervated mammalian skeletal muscle have been investigated extensively with a view to understanding the molecular organization of the sarcolemma. Denervation has been employed in order to understand the molecular alterations of the sarcolemmal membrane in relation to the known development of hypersensitivity of this membrane to acetylcholine (Ach) resulting from denervation. The morphological, physiological and biochemical alterations of mammalian skeletal muscle subsequent to denervation have been reported previously. However, the significance of these alterations in the structure of the sarcolemma is not fully understood. The particular aspects of the sarcolemma that require investigation concern the alterations in acetylcholinesterase (AchE) activity and receptors for Ach in denervated muscle. Both AchE and receptors for Ach are bound to the sarcolemma and therefore this membrane has been investigated by using techniques that are suited to investigate the structure of the cellular membranes. Such techniques include histochemical demonstration of AchE at electron microscope level and freeze-fracturing. The technique of freeze-fracturing enables a direct visualisation of the interior of cellular membranes. This latter feature is not accessible by any other known technique. Apart from the sarcolemma of the denervated muscle, alterations in the cellular structure of the muscle as well as the degeneration of the nerve terminal have been studied by electron microscopy. The innervated contralateral lumbricals have been used as controls.

Acetylcholinesterase localisation at the level of electron microscopy has revealed a non-uniform localisation of enzyme activity in the synaptic clefts of denervated muscle. This is in contrast to a uniform localisation of enzyme activity in the synaptic cleft of innervated muscle. In the light of existing biochemical evidence, the non-uniform localisation of AchE is considered to be due to loss of this enzyme in denervated muscle, and the loss is assumed to be due to one (16s) of the three molecular forms of AchE (16s, 10s, 4s) which is known to undergo the largest decrease upon denervation.

The freeze-fracturing data show that the convex face (P) of the innervated non-synaptic sarcolemma has random distribution of 80 A^{o} particles with a packing density of 2000/μ^2. The concave face (E) shows far fewer of such particles. As a result of denervation, the macromolecular organization of the convex face (P) undergoes a marked change. There is a noticeable loss of smaller particles (80 A^{o}) and an increase in number of large particles (150-180 A^{o}). The packing density of these large particles is 400-1000 particles/μ^2 and their distribution varies in P faces of sarcolemma. It is hypothesized that these large particles represent Ach receptors. This assumption is based on the following rationale: 1) The size of the large particles in P faces of non-synaptic sarcolemma of denervated muscle is the same as those observed in the corresponding faces of synaptic sarcolemma. 2) The physiological and biochemical studies have shown that the non-synaptic Ach receptors increase in number after denervation and the large particles (150-180 A^{o}) are seen to be increased in number on P faces of non-synaptic sarcolemma only after denervation. The different distribution patterns of these

large particles on P faces of non-synaptic sarcolemma may reflect gradual and progressive changes. The identity of these large particles on P faces of non-synaptic sarcolemma of denervated muscle requires confirmation by labelling of the receptor and freeze-etching process.

Apart from the above studies on sarcolemma, observations have been noted on the alterations in the cellular structure of degenerating nerve terminal and muscle. These include: 1) Engulfment of nerve terminals by Schwann cell within 24 h after denervation. 2) The necrotic changes in the muscle include the disruption of myofilaments, increase in lysosomes, membrane whorls, and lipid globules. There is an increase in ribosomes and rough endoplasmic reticulum which may be partly related to the synthesis of extrajunctional Ach receptors that are known to increase in number in non-synaptic sarcolemma of denervated muscle.

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ABBREVIATIONS

The following abbreviations have been used in this thesis:

Acetylcholine	=	Ach
Acetylcholinesterase	=	AchE
Angstrom	=	A^{o}
α-Bungarotoxin	=	α-BGT
β-Bungarotoxin	=	β-BGT
Cholinesterase	=	ChE
Difluorophosphate	=	DFP
Hour	=	h
Micron	=	μ
Ribonuclease	=	RNAse
Tetrodotoxin	=	TTX

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INTRODUCTION

This thesis deals with an investigation of the denervated skeletal muscle with a particular emphasis on the sarcolemma, from a view to correlate the structure with its function. The rationale for using sarcolemma as a model system is the following: 1) Our present understanding of its functioning is much better than many other membrane systems. The sarcolemma is clearly compartmentalized into synaptic and non-synaptic zones. The synaptic region is characterized by the predominance of acetylcholine (Ach) receptors (Porter *et al.*, 1973; Fertuck and Salpeter, 1974) and acetylcholinesterase (AchE) involved in transmitter binding and its hydrolysis, respectively. The binding of transmitter to Ach receptors increases the permeability of the sarcolemma to Na, K and Ca leading to generation of synaptic potential that in turn initiates action potential spreading over the entire muscle membrane (Rang, 1974). 2) It is known from physiological and biochemical studies in innervated muscle that the sensitivity to Ach is restricted in the synaptic region (Axelsson and Thesleff, 1959) where Ach receptors are concentrated (Fertuck and Salpeter, 1974). In innervated muscle, the density of extrajunctional Ach receptors is very low. However, following denervation, the number of these receptors in the extrajunctional region increases (Hartzell and Fambrough, 1972) with a parallel increase in Ach sensitivity in non-synaptic regions (Axelsson and Thesleff, 1959). Therefore, denervation offers a convenient system of producing these receptors and studying their macromolecular organization in synaptic as well as non-synaptic regions.

According to the present concept, the biological membranes are composed of lipids, proteins (lipoproteins or glycoproteins) and carbohydrates. The lipids are amphipathic and are mostly arranged in a bilayer with their polar groups in contact with aqueous phase, while the non-polar hydrocarbon chains are sequestered away from the aqueous environment. The proteins associated with membranes are classified as peripheral and integral. The peripheral proteins are bound to the membrane by weak electrostatic interaction and can be removed by mild treatments like change in the ionic strength of medium. The integral proteins are closely bound to, and intercalated in the bilayer and they require drastic treatments with reagents such as detergents, protein denaturants or organic solvents to dissociate them from the membrane (Singer and Nicolson, 1972).

Currently, several biochemical and biophysical approaches that have been used in studies of membranes are circular dichroism, nuclear magnetic resonance, electron spin resonance, freeze-fracture-etching technique, autoradiographic labelling, fluorescent antibody technique, etc. For these approaches, several models of synthetic lipids and natural membranes have been used extensively.

In this investigation, the technique used for study of membrane is freeze-fracture. The apparatus for etching and replication in a vacuum was first made by Hall (1950). In the later years many other researchers like Steere (1957), Moor and Muhlethaler (1963), Bullivant and Ames (1966) modified the technique and the freeze-fracturing apparatus utilised for obtaining the replicas of biological material. This technique facilitates the fracturing process in the hydrophobic zone which is least resistant to freezing, and thus splits open the

membrane into an inner and an outer half. Therefore, by usage of freeze-fracturing technique, the internal structure of the membrane can be visualised which is not discernible by any other technique currently in use.

It has been mentioned above that in the present study the synaptic and non-synaptic regions of the sarcolemma are emphasized. Apart from freeze-fracturing, other techniques have also been used. These include thin sectioning, histochemistry for detection of carbohydrates, AchE and RNA. Denervation has been employed for the precise purpose of inducing alterations in the sarcolemma, so that an attempt can be made to correlate the structure with its functional organization.

MATERIAL AND METHODS

Material:

The rats used in these experiments are females belonging to the Sprague Dawley strain and weigh between 100-150 grams. The animals are anaesthetized by intraperitoneal injection of 1 ml of 5 mg/ml of sodium pentobarbital (approximately 30 mg/kg).

In many of the early denervation experiments, gastrocnemius diaphragm muscles, sartorius and extensor digitorum longus of the rat have been used (Miledi and Slater, 1969; Hartzell and Fambrough, 1972; Porter *et al.*, 1973). For the present investigation, lumbricals of the hind leg are chosen because of their small size and, therefore, they can be prefixed *in situ* relatively rapidly.

Denervation procedure:

The gluteus maximus and gluteus minimus in the pelvic region are exposed by excising a 2 cm square piece of skin. The facia covering the muscles is removed and the two muscles are separated by blunt dissection. The underlying sciatic nerve is lifted and approximately 1 cm of its portion is snipped off. After suturing the skin, the animals are returned to their respective cages. In all the experiments, the left leg of the animal is denervated and the contralateral right leg serves as a control.

Electron microscopy:

a) Thin sectioning

The animals have been sacrificed at intervals ranging from 6 h, 12 h, 24 h, 4 days, 8 days, 15 days and 2 months after denervation.

After anaesthetizing, 0.5-1 cc of cold (0-5^{o}C) 2% glutaraldehyde (buffered with 0.1 M phosphate, pH 7.2) is injected into the paw to fix lumbricals *in situ*, and 5-10 minutes later the skin on the ventral side of the paw is excised. Plantar aponeurosis with its adhering connective tissue is gently removed. The lumbricals are removed for further fixation in 2% buffered glutaraldehyde for 2 h. After rinsing in the same buffer as is used in the fixative, the muscles are post-fixed for another 2 h in 2% buffered osmium tetroxide. The muscles are rinsed in buffer for 20 minutes and then are dehydrated in a graded series of ethanol. Infiltration and embedding is done in an Araldite mixture consisting of equal volumes of Araldite 502, Dodecenyl succinic anhydride and DMP 30, which is an accelerator for polymerisation of the resin. The tissues are embedded and polymerised at 60^{o}C. The sections are mounted on a formvar coated grid and stained with 6% uranyl acetate in ethanol and lead citrate (Venable and Coggeshall, 1965).

For studying the changes at the motor end plate, the synaptic junctions are first identified in one micron thick sections, and thin sections are prepared from the relevant areas of the blocks.

b) Histochemical techniques

(i) Ruthenium red staining

This staining technique has been used to demonstrate extracellular materials like mucopolysaccharide (Luft, 1971). The tissues are fixed in 2% glutaraldehyde followed by fixation in 2% osmium tetroxide at room temperature. Both fixatives are buffered with 0.1 M cacodylate buffer (pH 7.2) and contained 0.25% ruthenium red. Processing of the tissues is done as described in section a) except that

the sections are examined without additional staining with uranyl acetate and lead citrate.

(ii) Ribonuclease (RNAse) digestion

After fixation for 1 h in cold (0-5°C) 2% glutaraldehyde buffered with 0.1 M phosphate at pH 7.4, the muscles are sectioned (50 μ) on Smith and Farquhar's tissue sectioner and incubated for 2 h at 37°C in a solution of ribonuclease (2.1 mg/ml) buffered with .01 M sodium phosphate at pH 7.0 (Gauthier and Schaeffer, 1974). The post-fixation is done in 2% osmium tetroxide. Processing of the muscle is done in the same way as described in section a). The sections were stained only with uranyl acetate.

Note: The observations of the above two histochemical techniques have been included with the results of thin sectioning studies.

(iii) Acetylcholinesterase (AchE) staining technique

The histochemical technique has been used on control and 15 days denervated muscles according to the method reported by Rash and Ellisman (1974). The rats are anaesthetized, denervated and sacrificed as described before. Initial fixation is done in ice cold 0.5% glutaraldehyde (buffered with 0.1 M cacodylate at pH 7.2) for 1 h. During removal of lumbricals, 2-3 rapid changes of cold fixative are given to ensure the retention of enzyme activity in the tissues. After rinsing in the above buffer, the muscles are chopped (25-50 μ thick) on Smith and Farquhar's tissue sectioner and then stained for AchE activity. The incubation medium contains the following constituents:

(1) 0.1% Acetylthiocholine iodide

(2) 0.02 M Sodium citrate

(3) 0.002 M Copper sulfate

(4) 0.0003 M Potassium ferricyanide

(5) 0.1 M Cacodylate buffer (pH 6.0)

For inhibiting the enzyme substrate reaction, the medium contains all the above constituents and the inhibitor eserine at 10^{-6} M concentration (Karnovsky, 1964). The sections are incubated for 30-45 minutes in medium with or without inhibitor and then post-fixed in 2% buffered osmium tetroxide. The tissues are processed for thin sectioning as described before in section a). The sections are studied unstained.

c) Freeze-fracturing technique

The animals are sacrificed at two weeks after denervation. The excised muscles are fixed in 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2. After fixation, the muscles are glycerated. For increasing the chances of getting fractured faces through neuromuscular junctions, some muscles after fixation were stained for AchE as described in section b). When the end plate regions become slightly brownish, the muscles are thoroughly washed with buffer. Small pieces from junctional regions are cut and glycerated. Small pieces of muscle are then frozen in liquid Freon 22. Pt-c replicas are made after fracturing at -100°C in Balzars BA 360 M high vacuum freeze-etch unit. The replicas are cleaned overnight in 40% chromic acid and examined in a Phillips EM-300 electron microscope. The original negatives have been magnified to 80,000 for measurement of intramembranous particle size.

Number of experiments carried out:

1. In each of the control and denervated group the results of thin sections and histochemistry are based upon the examination of 8-12 muscles from four rats.

2. In the freeze-fracture study, 60 replicas have been prepared from control muscles of 20 rats. Thirty-two replicas out of 10 denervated rats have been prepared. Out of these, 40 replicas of control muscle and 12 replicas of denervated muscle have been chosen for study.

RESULTS

I. CONTROL MUSCLE

The following description is based upon the study of lumbrical muscles of the rat, the structure of which is essentially very similar to that of white (fast-twitch) skeletal muscle described by Bloom and Fawcett (1975). The white muscle fibers are distinguished by the presence of few mitochondria, arranged in pairs or small rows, scanty ribosomes, and are larger in diameter as compared to those of red fibers (slow-twitch) which have abundant mitochondria and ribosomes. In lumbrical muscle of the rat most of the fibers appear to be of white type, although occasionally red fibers are encountered as well. In the following results only salient features of control muscles are described which are relevant to denervation experiments.

Electron microscopy:

a) Study of thin sections

(i) Structural organization of myofiber

An individual myofiber is covered by a basement lamina which is approximately 400 A^{o} thick (Fig. 1). It stains intensely with ruthenium red (Fig. 2) and is, therefore, concluded that it contains mucopolysaccharides. Collagen fibers often come into close association with the basement lamina. The sarcolemma is 80 A^{o} thick and it invaginates in the extrajunctional regions to form pinocytotic vesicles or the elements of the T-system (Figs. 1 and 2). The nuclei of the muscle cells are elongated and are always near the periphery (Fig. 3). The perinuclear cytoplasm is rich in ribosomes and cisternae of endo-

plasmic reticulum as compared to the rest of the non-junctional sarcolemma which has sparse ribosomes and rough endoplasmic reticulum (Fig. 1).

The bulk of the sarcoplasm consists of contractile elements organized into regular sarcomeres. The Z-line is 400-500 A^{o} thick and bisects I-band while M-line passes transversely through the middle of the A-band. The width of I-band is known to vary depending on the contractile state of the muscle. In the interfibrillar sarcoplasm there are elements of the sarcoplasmic reticulum, mitochondria and T-system (Fig. 3). Sarcoplasmic reticulum is seen as a network of tubules in the interfibrillar sarcoplasm. The mitochondria are found either in pairs opposite I-band (Fig. 3) or in small rows in the peripheral and interfibrillar sarcoplasm (Fig. 4). But in the vicinity of the blood vessel (Fig. 4) and neuromuscular junction, large conglomerates of mitochondria are seen between the sarcolemma and the outermost myofibrils. In longitudinal sections each sarcomere is seen to have a pair of triads located at the junction of A- and I-band (Fig. 5) When the plane of the section is parallel to the long axis of the triad it shows a central T-tubule about 200 A^{o} wide and sacs of terminal cisternae on either side of it. The membranes of the T-tubule and the terminal cisternae run parallel for a considerable distance (3000 A^{o} approximately). Running transversely across the space between these two membranes are electron dense lines which are 150 A^{o} apart (Fig. 6).

(ii) Neuromuscular junction

Presynaptic complex: The central portion of the muscle shows a large number of myelinated nerves between the muscle fibers. Each of

these nerve fibers consists of several myelinated axons enclosed in a connective tissue sheath, the epineurium. The cytoplasm of Schwann cells in which the myelinated axon lies is rich in ribosomes. The axoplasm reveals the usual organelles like mitochondria, smooth vesicles and thin neurofilaments (Fig. 7). As the nerve fiber approaches the muscle it is without a myelin sheath and the unmyelinated nerve fiber branches into terminal arborization. Each of these terminal branches is pear-shaped and shows characteristic synaptic vesicles 500-800 A^{o} in diameter apart from mitochondria and other organelles. The synaptic cleft is approximately 500-800 A^{o} wide (Fig. 8).

Post-synaptic complex: The motor end plate is approximately 10 μ wide. The motor nerve terminal abuts in the indented portion of sarcolemma which is highly folded. The folds are 5000-6000 A^{o} deep into the sarcoplasm. The juxtaneural portion of the sarcolemma appears thicker (160-200 A^{o}) as compared to 80 A^{o} in the non-synaptic regions of the sarcolemma (Figs. 8 and 9). The basement lamina is continuous and follows the invaginations of sarcolemma (Figs. 8, 9, 10). On the cytoplasmic side the sarcolemma is associated with fine fibrous material (Fig. 10). The soleplasm contains numerous fine microfilaments which seem to approach and contact the sarcolemma in some regions. Besides these microfilaments, the soleplasm is rich in ribosomes, profiles of endoplasmic reticulum and mitochondria (Fig. 8).

b) Acetylcholinesterase (AchE) localisation

(i) Validity of histochemical reaction

The localisation of AchE was studied by using a histochemical technique

given by Rash and Ellisman (1974) which, essentially, incorporates the details of the procedure introduced by Karnovsky and Roots (1964). The substrate used in the present histochemical study is Acetylthiocholine iodide which is hydrolysed only by Cholinesterase (ChE) (specific and non-specific). Therefore, it is considered to be a more specific substrate for localisation of AchE than thiolacetic acid (Zack and Blumberg, 1961; Barrnett, 1962) which is hydrolysed by esterases in general.

The enzymatic reaction starts with hydrolysis of thiocholine ester by ChE. Liberated thiocholine reduces ferricyanide to ferrocyanide, which then reacts with copper to give electron opaque insoluble precipitate of copper ferrocyanide. The redox potential of the couple cu/cu^{2+} is -0.27 volts relative to the hydrogen electrode, and that of $[Fe(CN)6]^{4-}/[Fe(CN)6]^{3-}$ is -0.44 volt. Therefore, the possibility of reduction of copper salt is far less than that of ferricyanide. Sodium citrate present in the incubation medium complexes with copper and thus prevents the formation of precipitate of copper ferricyanide, which can otherwise result from reaction of copper salt with potassium ferricyanide (Karnovsky, 1964).

(ii) Localisation of AchE

The reaction product seen as an electron dense deposit is uniformly distributed in the synaptic cleft. The motor nerve terminal does not show any electron dense deposit associated with the synaptic vesicles or other organelles in the axoplasm (Fig. 11).

Apart from synaptic clefts, the enzyme activity is seen to be associated with the axonal membrane of myelinated nerve fiber in non-uniform fashion (Fig. 12). In the muscle fibers, in only one instance,

a dense deposit has been noted in the muscle sarcoplasm (Fig. 13). The lack of enzyme activity in the muscle sarcoplasm should not be taken as an indication of the absence of ChE. The negative histochemical results in this study may be related to a slower rate of hydrolysis of substrate by ChE in sarcoplasm under the experimental conditions used in this study.

In the presence of eserine, no electron dense deposits have been detected in association with synaptic clefts, axonal membrane or muscle sarcoplasm (Fig. 14), thereby indicating that the electron dense deposits are the end product of the enzymatic reaction.

c) Freeze-fracture studies

(i) Identification of the fractured faces

It is well known that in freeze-fracturing technique the fracture plane passes through the hydrophobic zone which provides the path of least resistance. If the frozen material is etched, the water sublimes and the true surface of the membrane is exposed (Chalcroft and Bullivant, 1970; Branton, 1971). Therefore, freeze-etching provides a valuable technique for making direct observations of the interior of biological membranes, and it also enables visualisation of the true surface of the membrane. As shown in Figure 15, the inner face adjacent to the cytoplasm of the cell, and as viewed from outside, is a convex fracture and is often referred to as face P. The outer half of the membrane close to the cell exterior, when viewed from inside the cell, is a concave fracture and is referred to as face E (Branton *et al.*, 1975). The replicas prepared from these fractured faces of a plasma membrane show smooth areas and particulate areas. These intramembranous particles (referred to as particles) are generally 85 A^{o} in

diameter. Also, in the majority of the membranes studied, these particles seem to be attached preferentially to the face P and, therefore, this face has far more particles than those on face E (Fig. 16). This kind of asymmetrical organization is a common feature of biological membranes (Branton, 1971; Tewari and Malhotra, 1974; Bretscher and Raff, 1975; Malhotra *et al.*, 1975; Stoeckenius, 1976). The nature of these smooth areas and of the particles has been studied extensively. The smooth areas and the particles represent lipid and protein components of the membrane, respectively. These conclusions have been derived from some of the following observations:

1. When fatty acid bilayers are frozen and fractured the replicas show only extensive smooth areas. This suggests that the smooth areas of replicas made from biological membranes represent the lipid component (Deamer and Branton, 1967). Also, myelin, which contains 80% lipid and 20% protein when freeze-fractured, shows essentially smooth areas with a few particles (Branton, 1967; Malhotra *et al.*, 1975).
2. Digestion of red cell membranes (Branton, 1971) by a proteolytic enzyme, pronase, for 10 h results in the total loss of these particles on the membranes, suggesting that these particles on the fractured faces are proteinaceous in nature.
3. *Acholeplasma laidlawii* membranes from cells incubated in medium containing inhibitors for protein synthesis, puromycin, or deficient in amino acids, when freeze-fractured, show a decrease in number of particles on the fractured faces, thereby suggesting that the particles represent the protein component of the membrane (Tourtellote and Zupnik, 1973).

The number and distribution of these proteinaceous particles

varies depending on the functional state of the membrane. For example, relatively inert membrane such as myelin sheath appears as a smooth sheet with very few particles (Malhotra *et al.*, 1975), whereas membranes of chloroplast and mitochondria, which are relatively metabolically active, show an abundance of these particles (Branton and Park, 1967; Tewari *et al.*, 1973). Also, the macromolecular organization of a membrane may be different in different regions of the same cell. This is best demonstrated in organized epithelial cells of the kidney (Camilli *et al.*, 1974; Tewari and Malhotra, 1974).

(ii) Fractured faces of the sarcolemma

In the following description, the structure of the sarcolemma in the synaptic complex is described and compared with that of the non-synaptic complex.

Non-synaptic complex

P-face: The replicas of the rat lumbricals show extensive areas of the fractured P-face of sarcolemma. This face is characterized by the following features:

1) There are randomly dispersed depressions which are 300-500 A^{o} in diameter (Fig. 17). These are presumably fractures through the openings of the T-system or caveolae (Rash and Ellisman, 1974).

2) A large number of randomly dispersed intramembranous particles are present. These range in size from 40-180 A^{o}, although the 80 A^{o} particles are by far the most predominant. There are approximately 2000 particles/μ^2 on this fractured face (Fig. 17). This packing density of intramembranous particles is comparable to that reported for the corresponding fractured face of the sarcolemma in frog (Ishikawa *et al.*, 1975).

3) Some of these intramembranous particles form orthogonal arrays consisting of 8-10 particles. These particles are 50-60 A^{o} in diameter (Fig. 18).

4) Large particles of 150-180 A^{o} diameter are extremely few and occur in small groups (Fig. 19).

E-face: This face, which is complementary to the P-face, has the following distinguishing features:

1) There are protuberances that presumably correspond to the 300-500 A^{o} depressions on face P.

2) Intramembranous particles are far fewer on this face. They are mostly 80 A^{o} in diameter and have packing density of 200 particles/μ^2 (Fig. 20).

Ideally speaking, the E-face should have depressions to match the particles on the complementary P-face, but such depressions singly or in groups were not seen in the replicas. The absence of these depressions has been noted by many researchers (Flower, 1973; Malhotra and Tewari, 1973) and may be due to one or more of the following reasons:

(i) Plastic deformation of the membrane during fracturing.

(ii) Contamination of the depressions (Flower, 1973).

(iii) Filling of depressions during shadowing (Flower, 1973).

(iv) Preferential attachment of the particles to P-face (Stockenius, 1976).

Synaptic complex

It should be emphasized at the outset that large areas of the fractured sarcolemma in the synaptic complex have not been detected in freeze-fracture replicas. The study of approximately 40 replicas

made out of 20 experiments done on control muscle resulted in only two replicas showing fractures through the synaptic complex. This is perhaps due to the small size of the synaptic region as well as to the inability of the fracture plane to follow through the deeply invaginated sarcolemma in the synaptic complex. Therefore, the following observations are based on a small number of fractures through the post-synaptic sarcolemma. The synaptic complex can be easily identified in cross-fractures which are comparable to the appearances of synapses in thin sections (Figs. 21 and 22). Such fractures have been used to facilitate identification of the fractured faces of the post-synaptic sarcolemma. The fractured faces are distinguished from the corresponding faces of the non-synaptic region by the following features:

P-face:

1) There are rows of particles which are 150-180 A^{o} in diameter. These particles are often seen in the upper region of the folds and have a packing density of 1800/μ^2 (Fig. 23) which is comparable to that described for this face in extensor digitorum longus gastroenemius and diaphragm muscles of the rat (Rash and Ellisman, 1974).

2) There are no depressions on this face corresponding to those of T-system or caveolae on the P-face of non-synaptic sarcolemma.

3) The smaller particles of 80 A^{o} diameter are found only in the deeper region of the folds (Fig. 23).

E-face:

1) It has a pitted appearance.

2) It has very few particles (Fig. 23).

3) Protuberances (300-500 A^{o}) which are present on the corres-

ponding fractured face of non-synaptic sarcolemma are not observed on this face in the synaptic region.

4) There are no depressions (Fig. 23) corresponding to the particles on the P-face and reasons for their absence have already been elaborated on page 16.

II. DENERVATED MUSCLE

Gross changes:

The denervated toe loses its nervous response immediately after the sciatic nerve is sectioned. The excised experimental muscles show a slight increase in weight up to 4 days followed by a continuous decrease in their weights. By 15 days the excised muscles weigh approximately 75% of the control weight (Fig. 24). By this time the diameter of each individual muscle fiber is smaller (15 μ) than that of the control muscle fiber (30 μ). The muscles also appear more pale as compared to the controls.

Electron microscopy:

a) Study of thin sections

(i) Changes in the presynaptic complex

A study of 30 synaptic junctions of denervated muscle reveals that there is a considerable variation in the degenerative process at the nerve terminal. Mostly, the changes start to be seen 6 h after the denervation. The axoplasm sometimes shows small membrane whorls not apparent in the controls (Fig. 25). The synaptic vesicles that are randomly dispersed in the control nerve terminal tend to clump in degenerating nerve terminals (Fig. 26).

Twelve hours after denervation the Schwann cells appear to surround the nerve terminals (Fig. 27a) and cause fragmentation of the latter (Fig. 27b). In some regions the Schwann cell processes appear to follow the profile of the nerve terminal and come to intervene between muscle cell and the nerve terminal (Figs. 27a and 29).

Within the axoplasm of some nerve terminals, the clumping of synaptic vesicles becomes more evident (Figs. 27a, b), while there are also indications that their number decreases in others (Figs. 28 and 29). Mitochondria also undergo degeneration as they appear greatly swollen in some nerve terminals (Figs. 27b and 28). Occasionally a membranous vesicle is seen in the synaptic cleft (Fig. 29) and it resembles the synaptic vesicle, which may escape if the nerve terminal is damaged (Fig. 29).

One day after denervation the nerve terminals disappear and the Schwann cells lie in the region previously occupied by the nerve terminals. The cytoplasm of Schwann cells at this time shows electron-dense material which presumably represents the phagocytosed contents of axon terminals (Figs. 30a, b). Schwann cells seen in this region disappear 4-8 days after denervation.

(ii) Changes in the myofiber

In muscle cells the process of degeneration occurs seemingly at random because different muscle cells show different levels of change in their structure subsequent to denervation. The following description, therefore, provides a generalised account of muscle necrosis.

Post-synaptic complex: During the period when the nerve terminal undergoes degeneration and disappears, i.e. 24 h after denervation, the only detectable change seen in the post-synaptic region is the loss of bulb-like indentations of the sarcolemma. The folds are retained for a long time and are noticeable even 15-20 days after denervation. However, they appear to decrease in their depth as early as 2 days after denervation (Fig. 31), and by 15 days there is a noticeable reduction in their number (Fig. 32). In the soleplasm, cyto-

plasmic organelles identifiable as lysosomes in electron micrographs are of common occurrence as early as 2 days after denervation. The lysosomes are in the form of multivesicular bodies or membrane bound vesicles with amorphous contents (Figs. 31 and 34). In contrast to the denervated muscle, lysosomes have not been seen in the control muscle cell. The microfilaments continue to maintain contact within post-junctional sarcolemma but they seem to be shorter in length as compared to those observed in controls (Fig. 33). This may, however, be due to their disorientation subsequent to denervation. Besides the occurrence of dispersed microfilaments, bundles of microfilaments (Fig. 34) become apparent in the soleplasm 2 days after denervation. Such aggregated microfilaments do not occur in the control muscle cell.

Non-synaptic complex: The changes due to denervation are first noticeable in the peripheral sarcoplasm, there being no apparent change in the appearance of the basement lamina (Fig. 35). In the peripheral region the myofilaments appear disrupted and scattered within 2 days after denervation (Figs. 36 and 37). The myofilaments in the interior of muscle cell are stable even 15 days after denervation and their disruption is only a rare event. But 2 months after denervation the myofilaments lose their regular pattern of striation and, in longitudinal sections, transversely as well as longitudinally oriented myofilaments can be seen (Figs. 38 and 39). In the regions where myofilaments are disrupted, Z-band appears fragmented and disoriented (Figs. 38 and 39).

Two days after denervation the peripheral sarcoplasm shows an increase in the number of lysosomes and smooth surfaced endoplasmic

reticulum (Fig. 40). Lysosomes are seen as multivesicular bodies or vesicles with amorphous content. Eight to 15 days after denervation, however, lysosomes are seen in the interfibrillar sarcoplasm as well (Fig. 42).

Membrane whorls consisting of 8-20 lamellae are seen in the peripheral sarcoplasm 2-4 days after denervation (Figs. 41 and 42), but 8-15 days after denervation they are of common occurrence in the interfibrillar sarcoplasm opposite I-band.

In addition to the above changes, vesiculation of the sarcoplasm is of common occurrence (Figs. 43 and 44). These vesiculated areas contain finely granular material. In one instance the nucleoplasm also showed vesiculation (Fig. 45). The peripheral alterations of the sarcoplasm are noticed in the early stages of denervation and may reflect the traumatic changes due to denervation.

Eight to 15 days after denervation the peripheral sarcoplasm shows an abundance of electron opaque particles, approximately 150 A^{o} in diameter, and an increase in the profiles of the endoplasmic reticulum. The free electron opaque particles, as well as those attached to the endoplasmic reticulum, are of similar size (Fig. 47a). Digestion with RNAse results in partial loss of these free granules and those attached to the endoplasmic reticulum (Fig. 47b), thereby indicating their ribosomal nature. In contrast to these observations on the denervated muscle, there are very few ribosomes or profiles of endoplasmic reticulum in the peripheral sarcoplasm of the control muscle.

Two months and fifteen days after denervation there is a noticeable increase in membrane bound round bodies 0.5 μ in diameter, which are probably lipid globules. These are mostly located opposite I-band

and are often found in association with mitochondria (Figs. 48 and 49).

In denervated muscle, mitochondria lose their characteristic paired arrangement seen at the Z-band in the control. After prolonged denervation (2 months), some regions of the sarcoplasm show large aggregates of mitochondria (Fig. 50).

b) Acetylcholinesterase localisation

The distribution of enzyme activity, when visualised in electron micrographs, is not uniform, since electron dense deposits found in synaptic clefts are in patches (Fig. 51). It is emphasized that frequently post-junctional complexes are seen devoid of any electron dense deposits (Fig. 52). This variation in localisation of enzyme activity does not seem to be due to discrepancy in technical variation since in control muscles the enzyme activity is always uniformly distributed. Therefore, this variation in the denervated muscle is related to the general decrease in the AchE activity along the synaptic clefts.

In muscles incubated in a medium containing inhibitor, the enzyme activity is not detected anywhere in the synaptic clefts (Fig. 53).

c) Freeze-fracture studies

There is no detectable change in the extent of the fractured faces of the membranes available for study. All the replicas of denervated muscle have been made at the end of two weeks, because this period of denervation is known to correspond to the peak of extra-junctional sensitivity to acetylcholine in skeletal muscle (Hartzell and Fambrough, 1972). In the following description, therefore, alterations in the non-synaptic sarcolemma, resulting from denervation, are elaborated. Only scanty data is so far available on post-synaptic

membrane of denervated muscle, as only one out of 12 replicas showed fractures through this zone.

(i) Fractured faces of denervated sarcolemma

Non-synaptic complex

P-face: This face of the denervated sarcolemma reveals marked alterations as compared to that in the corresponding face of the sarcolemma in controls (Fig. 54). The alterations are of the following kind:

1) The orthogonal arrays are not visualised.

2) There is an apparent decrease in the number of smaller (approximately 80 A^{o}) particles but an increase in the number of larger particles (150-180 A^{o}) on this fractured face.

3) The distribution of particles is highly variable on this face, and in general 3 categories of distribution patterns are arbitrarily distinguishable:

Type I - This distribution is characterized by the presence of a few 80 A^{o} particles and many more 150-180 A^{o} particles. The packing density of the particles varies from 400-1000/μ^2, and is made up of predominantly large particles (Fig. 55).

Type II - This distribution is characterized by the presence of many aggregates of particles which are mostly 150-180 A^{o} in diameter. In each aggregate there may be 4-20 particles (Fig. 56).

Type III - Here there are only one or two aggregates of particles. These particles are large (150-180 A^{o}) and consist of 30-50 or more particles. The rest of the membrane has randomly dispersed particles (Fig. 57).

It is emphasized that distribution patterns of the large particles (150-180 A^{o}) described above have not been detected on the corresponding fractured face of the control sarcolemma (Fig. 54).

E-face: This face of the denervated sarcolemma in the non-synaptic zone shows no obvious alterations in structure from that described for the corresponding face of the controls (see Fig. 20). There are no depressions complementary to the distribution of particles on the P-face of Types II and III. The reasons for the absence of depressions corresponding to the particles on the complementary face have been mentioned previously (page 16).

Synaptic complex

The fractured faces of 15 days denervated synaptic sarcolemma do not show any alterations (Fig. 58) from the corresponding faces of synaptic sarcolemma of the control muscle.

The above data of thin sections, histochemistry, and freeze-fracture of control and denervated muscle have been summarised in comparative form in Table I. The results have been schematically represented in Figures 59 and 60.

DISCUSSION

The results of the present study on denervated muscle demonstrate the importance of the role played by the nerve in relation to the maintenance of the structural organization of the muscle fiber. It also shows that as a consequence of nerve transection there is disintegration of the nerve terminal, which is the initial reaction and is detectable by electron microscopy subsequent to denervation. The skeletal muscle, upon denervation, undergoes marked alterations involving initial transient gain in weight, atrophy of the muscle, a decrease in acetylcholinesterase (AchE) activity, and alterations in the non-synaptic sarcolemma. These results are discussed in the light of biochemical and physiological data available on the denervated skeletal muscle.

Following denervation, the muscles increase in weight by 70% and thereafter decrease and weigh only 75% of the control weight by 15 days. This phenomenon of transient gain in weight of denervated muscle has previously been reported in diaphragm muscles of the rat (Gutman *et al.*, 1966; Miledi and Slater, 1969). The initial gain in weight has been shown to be due to the increased uptake of amino acids by the denervated muscle and their incorporation into sarcoplasmic and contractile proteins (Buse *et al.*, 1965; Gutman *et al.*, 1966; Harris and Manchester, 1966). The loss in weight subsequent to the initial gain in denervated muscle has been demonstrated to be due to reduced incorporation of amino acids into the muscle proteins (Stewart, 1955; Helander, 1957). During atrophy of the muscle, not all of the muscle

proteins are affected similarly by denervation, since the contractile proteins exhibit decreased synthesis, whereas acetylcholine (Ach) receptors and proteolytic enzymes like aryl sulfatase, phosphatase, and cathepsins show enhanced synthesis (Gutman *et al.*, 1966; Syrovy *et al.*, 1966; Pollack and Bird, 1968; Brockes *et al.*, 1975). As the bulk of the skeletal muscle is made up by the contractile proteins, there is an overall decrease in weight subsequent to the transient gain in denervated muscle.

The present findings on the degenerative changes at the neuromuscular junction are in general agreement with the studies on the denervated muscles of the diaphragm and extensor digitorum longus of the rat (Miledi and Slater, 1963, 1968, and 1970); sartorius muscles of the frog (Katz and Miledi, 1959a, b; Birks *et al.*, 1959, 1960); and newt muscles denervated *in vivo* and cultured in the absence of nerve explants (Lentz, 1972). Even in experiments where the lumbrical muscles of mice have been denervated pharmacologically by injecting local anaesthetic, methyl bupivacaine, degenerative changes in neuromuscular junctions similar to those described in the present Results have been reported (Jirmanova, 1975).

In the present study it has been shown that the breakdown of the presynaptic ending is discerned approximately 12 h after denervation, and by the end of 24 h a total loss of the nerve terminal occurs. Electrophysiological studies on rat diaphragm muscle indicate that during the first 12 h most of the endings have the ability to transmit impulses. However, by 24 h the stimulation of the nerve which efficiently conducts action potential does not produce the electrical

excitation of the end plates indicating the failure of transmitter release (Slater, 1966; Miledi and Slater, 1970). The time at which the total structural breakdown occurs seems to vary depending on the type of animal used, experimental conditions, the kind of fiber (whether fast or slow) studied, and the distance of the transected nerve from the muscle. In frog muscle at 25°C, the failure in neuromuscular transmission occurs 3-5 days after denervation. Lowering the temperature to 10°C delays the failure by several days (Birks *et al.*, 1960). It is also known that failure of neuromuscular transmission in denervated fast fibers (with large motor axons) occurs earlier than the denervated slow fibers with smaller motor axons (Katz and Miledi, 1959b). It has been demonstrated that the longer the degenerating nerve stump, more is the delay in failure of neuromuscular transmission. For example, in rat diaphragm, sectioning of each additional centimeter of phrenic nerve has been shown to delay the failure of transmission by 45 minutes (Slater, 1966; Miledi and Slater, 1970).

The rapid degeneration of nerve terminal observed in this study is perhaps due to the interruption of nutrients synthesized in the cell body (Hofmann and Thesleff, 1972). The substances (proteins, carbohydrates, lipids) synthesized in the cell body are carried down the axon through axoplasmic transport at the rate of 410 mm/day (Ochs *et al.*, 1969; Ochs, 1972, 1974). In this study the notable structural breakdown of the nerve terminal is observed 12 h after denervation. This may mean that during the first 6-10 h after denervation the metabolites remaining in the axoplasm are being used up, and when these

are not replaced due to interruption of fast axoplasmic transport, the rapid breakdown of the nerve terminal occurs.

One of the features in degeneration of presynaptic complex is the involvement of Schwann cells in engulfing the nerve terminals. This is of common occurrence in vertebrates and has been noted not only in vertebrate nerve muscle synapse (Reger, 1959; Birks *et al.*, 1960; Jirmanova, 1975; Miledi and Slater, 1970) but also in synapses of the frog sympathetic ganglion (Hunt and Nelson, 1965) and in the vertebrate central nervous system (Colonnier, 1964; Gray and Guillery, 1966; McMahan, 1967). To date it is not known what causes the proliferation of Schwann cells. Miledi and Slater (1970) have hypothesized that a signal is produced at the site of injury and is passed down through axoplasmic flow, and in support of this suggestion is their finding that the longer the degenerating nerve in the stump, more is the delay in failure of neuromuscular transmission.

Many of the alterations in the denervated muscle that are noted in the present study have been reported previously by other workers (Pellegrino and Franzini, 1963; Pollack and Bird, 1968; Miledi and Slater, 1969). However, neither the increase in ribosomes in white muscle fiber nor the demonstration of reduced AchE activity at the level of electron microscopy have been reported. Furthermore, the alterations in the macromolecular organization of the non-synaptic sarcolemma of the denervated muscle have not been reported previously either.

It is generally agreed that, as a result of transection, the muscle undergoes degeneration which may be due to the loss of trophic

factor(s) (Hofmann and Thesleff, 1972), nerve activity (Salmons and Sréter, 1976), or muscle activity (Lomo *et al.*, 1974; Shainberg and Burstein, 1976). The mechanism underlying the degenerative changes of denervated muscle is not well understood so far. The results of this study show that the degenerative process of muscle starts after the nerve terminal is lost from the neuromuscular junction 24 h after denervation. The post-synaptic folds are visible even 15 to 20 days after denervation, although somewhat reduced in number and depth. While other explanations are feasible, it appears that the reduction in the post-synaptic folds is related to the transient weight gain in the initial period of denervation as well as to the lack of neural influence. During the initial stages of denervation, the area of the denervated muscle fiber is twice that of the contralateral innervated fiber (Miledi and Slater, 1969). Due to the sudden increase in size which results mainly from the increase in contractile proteins, the expansion of the cell occurs causing the sarcolemma to stretch, resulting in eventual reduction of the fold. Although in later stages of denervation the weight and size of the muscle decreases, the number and depth of folds is not returned to the control level, owing perhaps to lack of neural influence. It has been reported previously that invaginations of post-junctional sarcolemma occur in response to innervation (Lentz, 1970).

As the period of denervation increases, the muscle fibers become thinner compared to the contralateral controls. This reduction in size of thin denervated muscle has always been correlated with decrease in loss of contractile proteins in denervated muscle (Fisher

and Ramsey, 1946, 1949; Fisher, 1954). In the initial period of denervation, the changes are noticed in the peripheral region only. These are mainly in the form of focal vesiculation of sarcoplasm and disruption of myofilaments. These changes in peripheral sarcoplasm have been noticed in gastrocnemius, soleus and diaphragm muscles of rats (Pellegrino and Franzini, 1963; Gori, 1972; Tomaneck and Lund, 1973), and it has been suggested that they may indicate that the loss of contractile material occurs from the peripheral portion of the muscle (Miledi and Slater, 1969). In the later stages of denervation, however, the interior myofilaments get disrupted as well. The Z-line, which appears straight in longitudinally cut muscle of the control, appears wavy, fragmented, and disoriented in denervated muscle. These abnormalities of Z-line are also discerned in dystrophic human muscles with neurogenic atrophy (Engel, 1961; Shafiq *et al.*, 1967), in denervated gastrocnemius, soleus and diaphragm muscles of the rat (Pellegrino and Franzini, 1963; Miledi and Slater, 1969), in mouse muscle (soleus) paralysed by botulinum toxin (Duchen, 1971), and in mouse skeletal muscles (gastrocnemius and soleus) locally treated with tetanus (Duchen and Tong, 1973). The mechanisms underlying this disorientation and atrophy of myofilaments are not known, but it is observed that, concomitant with the focal vesiculation of the sarcoplasm and disruption of the myofilaments, there is a marked increase in the number of lysosomes. These organelles are seen near the periphery of the muscle cell in the initial stages of denervation, and are found in the interfibrillar sarcoplasm 15-20 days after denervation. The biochemical studies have shown that upon denervation there is increased protein

catabolism which seems to play the role in atrophy (Peter and Kohn, 1967; Goldberg, 1969). There is also an increase in hydrolytic enzymes like cathepsin, aryl sulfatase, and phosphatase in these lysosomes (Hajek *et al.*, 1964; Syrovy *et al.*, 1966; Pollack and Bird, 1968; Weinstock *et al.*, 1969). Subsequent to denervation, lysosomes of various forms such as multivesicular bodies and vesicles with amorphous contents appear. By histochemical techniques, alkaline phosphatase activity has been demonstrated in all these forms of lysosomes (Schiaffino and Hanglikova, 1972). However, morphological evidence of engulfment of myofibrillar components by lysosomes has not been seen. Therefore, it is possible that, following denervation, lysosomes may release hydrolytic enzymes that slowly digest the myofibrils.

Another feature of the denervated muscle is a notable accumulation of lipids and an increase in membrane whorls in the sarcoplasm. The accumulation of lipids is commonly observed in various muscle diseases (Shafiq *et al.*, 1967), and in pancreas and liver of rats subjected to toxic stimuli (like β-3 thienylalanine, an analogue of phenylalanine and azaserine, an inhibitor of purine biosynthesis) (Swift and Hruben, 1964). It is suggested that the possible source of the accumulated fats is the complex lipoprotein molecules which occur in cells (Adams, 1975). The membrane whorls have often been noted in human dystrophic muscle (Tome and Mair, 1970; Fisher *et al.*, 1972; Gambarelli *et al.*, 1974), in muscles of rats treated with colchicine (Markland and D'Agostine, 1971) or vincristine (Anderson *et al.*, 1967; Bradley, 1970). On the basis of electron microscopical evidence, Anderson *et al.* (1967) suggested that the membrane whorls on muscle

fibers of rats which are treated with vincristine originate from the preexisting membranes of sarcoplasmic reticulum which are altered in response to a change in environment.

The thin sectioning studies indicate that following denervation there is notable increase in free ribosomes and rough cisternae of endoplasmic reticulum in the subsarcolemmal region. Nerve sectioning has been reported to result in a conspicuous increase in ribosomes and endoplasmic reticulum in red fibers of rat semitendinous muscle (Gauthier and Dunn, 1973) and diaphragm muscle (Gauthier and Schaeffer, 1974). This investigation has shown that in white fibers which have sparse ribosomes and elements of endoplasmic reticulum in the control, a marked increase in these organelles occurs in response to denervation. These results, therefore, suggest that in denervated muscle there is new machinery for protein synthesis. It is known from previous studies that denervation leads to augumented synthesis of proteolytic enzymes (Pollack and Bird, 1968) and acetylcholine receptors (Brocks *et al.*, 1975). It is observed that lysosomes begin to appear in the sarcoplasm of denervated muscle long before there is a notable increase in ribosomes and endoplasmic reticulum. The increase of ribosomes and endoplasmic reticulum is observed only at a stage when there is an increase in Ach receptors in extrajunctional region of denervated muscle (Hartzell and Fambrough, 1972). Therefore, the increase in ribosomes or endoplasmic reticulum in the subsarcolemmal region may be partly related to the synthesis of Ach receptors in denervated muscle.

Amongst the proteins of the sarcolemma, the two most studied

are the AchE and Ach receptors that are associated with sarcolemma. The major role of AchE located in the post-junctional region is the hydrolysis of acetylcholine (Aidley, 1971). The electron microscopic studies on the purified molecular forms of AchE (8s, 14s, 18s) from *Electrophorus electricus* have shown that AchE is an elongated structure with a multisubunit head and a tail approximately 500 A^{o} long (Dudai *et al.*, 1973). The tail is probably a polypeptide and may play a role in anchoring the enzyme in the basement membrane (Silman, 1976).

In innervated muscle, the specific ChE (AchE) activity is highly concentrated at the synaptic cleft where it is detectable by histochemical (Couteuz, 1960; Eranko and Tetavainean, 1967; Barrnett, 1962; Rash and Ellisman, 1974) as well as autoradiographic techniques (Salpeter, 1967). The autoradiographic studies by incubation of excised rat sternomastoid muscles using tritiated DFP, which phosphorylates ChE, have shown that 85% of AchE activity is associated with the synaptic clefts, (Salpeter, 1967). The present investigation indicates that AchE reaction occurs at the end plate alone, since nonspecific enzyme activity has not been detected in the non-synaptic region. Therefore, a large proportion of this ChE activity in the muscle must be represented by AchE at the junctional region.

In rat diaphragm the three molecular forms of AchE have been shown to exist with different sedimentation constants of 4s, 10s and 16s. All three forms hydrolyse Ach. Out of these three forms, 16s

form exists only at the synaptic region and may correspond to the end plate enzyme, whereas 4s and 10s forms are distributed throughout the synaptic as well as in the non-synaptic region. Subsequent to denervation, all three molecular forms are decreased considerably, although the largest proportional decrease occurs in the activity of 16s form which is found only in the synaptic region (Hall, 1973). The marked reduction in AchE activity at the end plate of the denervated lumbrical muscles studied in the present investigation may then be due primarily to the decrease in 16s form of AchE. The results on AchE localisation of denervated lumbrical muscles reported herein are in agreement with those in AchE localisation in denervated muscles of newts (Lentz, 1972).

Quantitative studies of ChE in general in denervated sternomastoid muscle of the rat have shown that in both end plate and non-end plate regions ChE activity decreases between 50-70% within 3 days and very little change occurs thereafter (Guth *et al.*, 1964). In mouse, however, the decrease in ChE activity is much less compared to that seen in rat, and denervation produces only 25% decrease in ChE within 3 days and 35% decrease in 14-28 days (McCaman, 1966).

The presence of ChE at the end plate is dependent upon innervation. This has been demonstrated in chick and guinea pig. The individuals of both these species were divided into four groups. In the first group, the forelimb muscles of the chick and the hind limb muscles of the guinea pig were denervated. In the second group, the same muscles were denervated and the animals were injected with DFP which is known to block ChE localisation by phosphorylating it. In the third and fourth groups, the nerve innervating the muscles was

sectioned and subsequently sutured in order to obtain a speedy regeneration in both untreated (3rd) and DFP treated (4th) individuals. The animals were sacrificed 60 days later and histochemical localisation of ChE was carried out. The reaction was weakly demonstrable in the first group. In the second group of animals treated with DFP, ChE activity was absent in motor end plates. In both DFP treated and untreated individuals the reaction was consistently positive in the third and fourth groups, which demonstrates the importance of innervation in synthesis of ChE at the end plate (Filogamo and Gabella, 1966).

Another protein of the excitable membrane, i.e., Ach receptor, has received a good deal of attention. This is owing to the introduction of the toxin, α-Bungarotoxin (α-BGT), from various poisonous species of snake (Lee and Chang, 1966; Lee, 1972). α-BGT is a small polypeptide of molecular weight of 8000 (Miledi and Potter, 1971), and it has the advantage of binding specifically and irreversibly to the Ach receptors. The binding is considered specific and irreversible because it blocks the depolarizing action of Ach on muscle; and Ach analogues (e.g., succinylcholine, carbachol). Furthermore, Ach antagonists (e.g., tubocurarine) compete with α-BGT in binding reaction (Changeux *et al.*, 1970). The tritiated or iodinated preparations of α-BGT are used in autoradiographic localisation of the Ach receptors or in their isolation from the sarcolemma for biochemical and quantitative analysis (Barnard *et al.*, 1971; Miledi and Potter, 1971; Berg *et al.*, 1972). It is assumed that one molecule of α-BGT binds to one Ach receptor, and, on the basis of this assumption, the quantitative studies have shown that in mouse end plates of diaphragm and sterno-

mastoid muscle there are 12,500 and 46,000 binding sites/μ^2, respectively (Porter *et al.*, 1973; Fertuck and Salpeter, 1976). In rat diaphragm there are 13,000 binding sites/μ^2 in the junctional region (Fambrough and Hartzell, 1972) in contrast to 5 binding sites/μ^2 in the extrajunctional region (Hartzell and Fambrough, 1972). The Ach receptors are concentrated near the juxtaneural portion of the synaptic folds (Albuquerque *et al.*, 1974; Fertuck and Salpeter, 1974). In mouse sternomastoid muscle the major concentration of α-BGT binding sites (30,500/μ^2) occurs near the juxtaneural portions of the folds (Fertuck and Salpeter, 1976).

The present freeze-fracture studies indicate that particles of 150-180 A^0 diameter are present on the P-face of post-synaptic folds. These particles appear to be concentrated in the upper region of the folds. On the basis of autoradiographic experiments in which Ach receptors are shown to be in greater concentration near the juxtaneural portion of the folds (Fertuck and Salpeter, 1974, 1976), the particles seen in the upper portions of the synaptic folds are assumed to be Ach receptors (Rash and Ellisman, 1974).

The present freeze-fracture studies indicate that in denervated muscle the fractured faces of synaptic sarcolemma are unchanged. These results are in agreement with the biochemical estimation of α-BGT binding sites in denervated end plates of rat soleus muscle fibers in which it has been observed that there is no change in α-BGT binding sites even 10 days after denervation, and 60-70% reduction occurs in these binding sites only 4-6 weeks after denervation (Frank

et al., 1975).

Electrophysiological studies by microiontophoretic application of Ach have established that depolarization is produced when Ach is applied in the junctional region. However, subsequent to denervation, the sensitivity of the membrane to Ach begins to spread to the extrajunctional region. Within 10 weeks in frog sartorius muscle fiber and 2 weeks in rat diaphragm muscle the entire sarcolemma becomes responsive to Ach application (Axelsson and Thesleff, 1959; Miledi, 1960). This increase in Ach sensitivity is correlated with an increase in the number of extrajunctional Ach receptors. The ^{125}I α-BGT labelled membrane preparation of extrajunctional regions of the denervated muscle shows that there are 1695 receptors/μ^2 in this region compared to 5 receptors/μ^2 in the regions of the innervated muscle (Hartzell and Fambrough, 1972). The extrajunctional and junctional Ach receptors share many properties in common while there are some differences indicating that the extrajunctional and junctional receptors are closely related though distinct molecules (Table II).

In the present studies it has been observed that the structure of the fractured faces of the non-synaptic sarcolemma of denervated muscle undergoes a marked change. The P-face shows a marked decrease in the number of smaller particles and an increase in the large particles (150-180 A^0). It is suggested that these large particles (150-180 A^0) in non-synaptic sarcolemma of denervated muscle are Ach receptors. This hypothesis is based on the following rationale: 1) The size of the large particles on the P-face of non-synaptic sarcolemma of denervated muscle is the same as those on the corresponding face of synaptic

sarcolemma. 2) These large particles increase in number on the P-face of the non-synaptic region only after denervation. Several lines of biochemical evidence have already indicated that the multiplication in receptor sites in the non-synaptic region of denervated muscle is due neither to the mobilization of Ach receptors nor to the activation of latent receptors, but is due to synthesis of new receptors (Fambrough, 1970; Gramp *et al.*, 1972; Brockes *et al.*, 1975; Devreotes and Fambrough, 1975). This has been demonstrated by incubation of denervated muscle in a medium containing ^{35}S-methionine which resulted in the appearance of labelled Ach receptors in the extrajunctional region (Brocks and Hall, 1975). Also, the inhibitors of protein synthesis, such as actinomycin D, Cycloheximide, and puromycin, are known to arrest the synthesis of new Ach receptors in the extrajunctional region (Fambrough, 1970; Gramp *et al.*, 1972). Apart from the demonstration of increase in number of large particles (150-180 A^{o}, hypothesized to be Ach receptors), the present freeze-fracture data further demonstrate that the distribution of these particles is variable over the non-synaptic sarcolemma. This is in contrast to innervated non-synaptic sarcolemma where the particles are always distributed in uniform fashion. It is, therefore, further proposed that the P-face of non-synaptic sarcolemma of denervated muscle undergoes rapid changes, and the different distribution patterns of large particles may reflect gradual and progressive changes in the membrane.

It is believed that the Ach receptors span the entire width of the membrane (Changeux *et al.*, 1975). This conclusion is based on the following experiments: 1) When the receptor rich membrane preparation

of *Torpedo marmorata* is negatively stained, the surface of the membrane appears particulate. When these membrane preparations are subjected to freeze-etching then particles (100 A^0) can be visualised at the true surface as well as on the P-face (Cartaud *et al.*, 1973; Nickel and Potter, 1973). 2) The receptor protein can be removed only by detergent treatment (Changeux *et al.*, 1970) and in solution it displays hydrophobic properties (Meunier and Changeux, 1973) indicating that it is an integral protein of the membrane. If the large particles seen on the P-face of denervated sarcolemma are Ach receptors themselves, then it should be possible to visualise them at the surface by the labelling and etching process (Hourani *et al.*, 1974). Studies of this nature are already planned as a continuation of the present investigation.

The structural organization of the muscle cell undergoes marked alterations following denervation. Various experiments have brought out three sets of ideas that attempt to explain the underlying causes behind alterations observed in denervated muscles. These are: 1) trophic factors released by neurons; 2) nerve activity and/or 3) the activity of the muscle itself. None of these ideas are mutually exclusive, and the question as to what specific factor accounts for the observed changes in denervated muscle is not resolved as yet. In the following discussion, therefore, an attempt is made not to support any particular idea or to solve the problem but to describe only a few experiments that are in favour of each of the three ideas mentioned above.

A wide body of existing evidence suggests that motoneurones release a substance(s) termed a trophic factor(s) that is transported

through axoplasmic flow. This trophic substance(s) probably maintains the structural organization of the muscle (Harris and Thesleff, 1971; Hoffman and Thesleff, 1972; Hoffman and Peacock, 1973). This opinion is based on experiments using drugs like colchicine and β-Bungarotoxin, which are known to interfere with axoplasmic flow. When colchicine is injected under the perineurium of the sciatic nerve of the rat, from 1 to 6 days later an increase in extrajunctional Ach sensitivity and the appearance of tetrodotoxin (TTX) resistant action potentials in innervated extensor digitorum muscles of rats have been reported (Hofmann and Thesleff, 1972). β-Bungarotoxin (β-BGT), a neurotoxin from *Bungarus multicinctus*, is known to block release of Ach from the motor nerve terminal. When hind legs of rats are injected with this toxin there is a complete paralysis of the muscle accompanied by an increase in extrajunctional cholinergic sensitivity and TTX resistant action potentials (Hofmann and Thesleff, 1972). These effects are presumed to be due to interference in axoplasmic transport by colchicine and in the release of acetylcholine along with other trophic substances by β-Bungarotoxin.

It is known also that in denervated muscle the longer the nerve stump the more prolonged is the trophic influence, thereby supporting further the hypothesis of a trophic factor(s) (Harris and Thesleff, 1972).

In relation to the influence of impulse activity, many researchers believe that the biochemical and contractile characteristics of the muscle are determined by the type of its innervation. Many years

ago Eccles *et al.* (1958) reported that slow muscle fibers are innervated by tonic motoneurones discharging at a rate of 10-20/sec, whereas fast muscle fibers are innervated by phasic motoneurones that have a high discharge rate between 30 and 60/sec.

It is known also that slow and fast muscles differ in their contractile and biochemical characteristics. For example: 1) The slowly contracting red muscles are preponderant in oxidative fibers mixed with a few glycolytic fibers, whereas rapidly contracting white muscles are rich in glycolytic fibers and have the substrates stored in the form of glycogen (Guth and Samaha, 1969). 2) The myosin ATPase of the oxidative fibers is acid stable and alkali labile, whereas that of glycolytic fibers is alkali stable and acid labile (Samaha *et al.*, 1970a, b). 3) The myosin ATPase of each of these fibers has distinct subunits that can be separated and characterised by electrophoresis (Samaha *et al.*, 1970). 4) Myosin or myofibrillar ATPase from fast twitch muscles of the cat can split ATP more rapidly than that of myosin or myofibrillar ATPase from slow twitch muscles (Buller *et al.*, 1969).

In cross-innervation experiments, slow muscle is innervated with a nerve which previously supplied the fast fiber and vice versa. In various cross-innervation studies it has been demonstrated that the above mentioned contractile and biochemical properties of slow and fast muscles are reversed (Buller *et al.*, 1960; Buller and Lewis, 1965; Buller *et al.*, 1969; Samaha *et al.*, 1970; Buller *et al.*, 1971). Previously, the reciprocal changes in the speed of contraction brought about by cross-innervation of fast and slow muscles were attributed to

the trophic factor(s) carried by motor nerves (Buller *et al.*, 1960). Now it generally is believed that these changes after cross-union are due to the change in patterns of impulse activity reaching the slow and fast muscles (Sréter *et al.*, 1973; Salmons and Sréter, 1976). It has been demonstrated experimentally that if motor nerves supplying fast muscles (tibialis anterior and extensor digitorus longus) of rabbit are stimulated at a frequency of 10^{Hz}/sec (which is close to the firing frequency of slow muscle), the contractile speed of fast muscle approaches that of slow muscle. Furthermore, electrophoretic pattern reveals that a fast muscle light chain pattern of tibialis anterior has changed on stimulation to one that contains two additional bands corresponding to those in soleus myosin. The light chain pattern of the stimulated fast fiber therefore contains five bands and is the same as that produced by coelectrophoresis of fast and slow muscle myosin (Sréter *et al.*, 1973). In another experiment, Salmons and Sréter (1976) supplied the slow muscle with a motor nerve from fast fiber. Eight weeks later in some animals the soleus is stimulated by a train of impulses at 10 Hz delivered through new innervation. The pattern of activation, therefore, is the same as that prior to cross-union. In other animals, the cross-united soleus is not stimulated. The results of these experiments show that the contractile speed and electrophoretic pattern of myosin in muscles which were cross-reinnervated and stimulated do not differ from the controls. The characteristics of the cross-reinnervated and unstimulated soleus, however, are similar to those of fast muscle fibers. These results, therefore, demonstrate that the reciprocal changes observed in cross-united muscles are due

to a change in the pattern of impulse reaching the muscle. Whenever the change in the pattern of impulse does not occur in cross-united muscles, the cross-union is ineffective.

Apart from the trophic factor(s) and the nerve activity, the muscle's own activity has been demonstrated to be responsible for changes in Ach sensitivity, AchE activity, and the twitch responses of the muscle (Drachman and Witzke, 1972; Lomo and Rosenthal, 1972; Shainberg and Burstein, 1976). It is commonly observed that upon stimulation of denervated muscle carrying high levels of extrajunctional Ach receptors, suppression of both the extrajunctional Ach sensitivity and the number of Ach receptors occurs (Drachman and Witzke, 1972; Shainberg and Burnstein, 1976). The increase in binding of ^{125}I α-BGT in the extrajunctional region also has been observed in diaphragm muscles of rats in which the neuromuscular blockade is produced by intraperitoneal injections of d-tubocurarine, succinylcholine or α-Bungarotoxin (Berg and Hall, 1975). In developing muscles of 11 day chick embryos, levels of AchE are high and the electrical stimulation of the muscles results in reduction of AchE activity of the cells (Walker and Wilson, 1975). Various experiments in which the muscles are directly stimulated have indicated that some of the differences in properties of fast and slow muscles are due to the differences in the pattern of muscle activity (Lomo *et al.*, 1974; Lomo and Westgaard, 1975a, b). This is demonstrated in denervated rat soleus muscle fibers. In one group of rats the denervated soleus muscle fibers are stimulated at 100 Hz for 0.5 second and the stimulation is repeated every 25 seconds. This type of brief stimulation is intended

to resemble the phasic activity of fast muscles. In the second group the denervated soleus muscle fibers are stimulated at 10 Hz. The stimulus lasted for 10 seconds and was repeated every 50 seconds. The longer lasting periods of 10 Hz stimulation resemble more the tonic activity of slow muscles. The results of this experiment show that muscles stimulated at 100 Hz have the twitch responses characteristic of fast muscle fibers. The histochemical analysis of actomyosin ATPase of these fibers (100 Hz stimulation) indicates that these fibers are darkly stained like those of fast muscle fibers.

The muscles stimulated at 10 Hz exhibit the characteristics of slow muscle fibers (Lomo *et al.*, 1974). The main difference with respect to Ach sensitivity is that many slow fibers have a low level of Ach sensitivity in extrajunctional regions, whereas there is no detectable sensitivity in fast muscles (Miledi and Zelená, 1966). If the denervated soleus muscles of rat are directly stimulated at 100 Hz for 1 second and every 100 seconds, the Ach sensitivity is reduced to 1 mv/nc within 4 days. On the other hand, if the denervated soleus is stimulated at 10 Hz for 10 seconds and every 100 seconds with continuous 1 Hz stimulation, in about 4 days the sensitivity to Ach is as high as in denervated and unstimulated muscle and 6-10 days are required to bring down the Ach sensitivity within normal range (Lomo and Westgaard, 1975b). In this experiment, the brief period of 100 Hz stimulation resembles somewhat the phasic activity of fast muscle, while the longer lasting periods of 10 Hz resemble more the tonic activity of slow muscle (Fischbach and Robbins, 1969). On the basis of these experiments, Lomo and Westgaard (1975a, b) suggested that the presence of

low but detectable Ach sensitivity in slow fibers and its absence in fast fibers (Miledi and Zelená, 1966) is related to the characteristic pattern of activity in the two types of muscles.

In addition to the muscle's own activity, some factor from the degenerating nerve terminal also may be responsible for Ach hypersensitivity in the denervated muscle. This has been demonstrated experimentally by double innervation of the muscle. The soleus is dually innervated by transplanting the fibular nerve onto the surface of the muscle. The soleus nerve is crushed 3 days after the transplantation so that a large number of soleus muscle fibers may be innervated by the fibular nerve and later become innervated by its own soleus nerve. In this dually innervated soleus, the end plates associated with the fibular nerve are in the proximal part, whereas those belonging to the soleus nerve are in the middle of the muscle fibers. It is observed that when the fibular nerve is sectioned, the development of hypersensitivity to Ach occurs in the proximal part of the muscle fibers which are innervated by the soleus nerve. After two weeks the surrounding hypersensitivity disappears, leaving only focal sensitivity at the degenerating end plates (Lomo and Westgaard, 1975b). These experiments, therefore, indicate that the muscle's own activity as well as the release of an unknown factor(s) from the degenerating nerve terminals are responsible for the Ach hypersensitivity of the denervated muscle.

In conclusion, in the present investigation an attempt has been made to shed light on the alterations of synaptic and non-synaptic regions of denervated muscle and correlate them with known physiologi-

cal and biochemical aspects. By the use of thin sectioning, histochemical, and freeze-fracturing techniques, many observations were made. Of these, the more significant ones are: 1) Upon denervation there is considerable increase in rough endoplasmic reticulum and ribosomes in peripheral sarcoplasm. This increase in endoplasmic reticulum and ribosomes occurs 8-15 days after denervation when the extrajunctional sarcolemma is known to be highly sensitive to Ach. Therefore, it is likely that the observed increase in endoplasmic reticulum and ribosomes may be related to the increased synthesis of extrajunctional Ach receptors in denervated muscle. 2) The histochemical studies on localisation of AchE show that the enzyme activity is either absent or is seen in patches in the synaptic cleft of denervated muscle. This is considered to be due to loss of AchE from the synaptic cleft of denervated muscle. 3) The P-faces of denervated sarcolemma show large particles (150-180 A^{o}). This is in contrast to the P-face of innervated sarcolemma which always has randomly dispersed 80 A^{o} particles. Based on physiological, biochemical and morphological evidence, it is hypothesized that these large particles on the P-face of denervated sarcolemma represent the Ach receptors. Further experimental evidence by labelling of the Ach receptor and the freeze-etching process is necessary to test the above hypothesis.

TABLE I. Summary of comparison between control and denervated lumbrical muscle

Thin sectioning, histochemical and freeze-fracturing studies

(only those features that show alterations are mentioned in the Table)

FEATURES INVESTIGATED	CONTROL	DENERVATED
Gross changes		There is an initial increase in weight during the first 4 days after denervation followed by a decrease. By 15 days the muscles weigh only 75% of the control weights.
Average diameter in the central region of the fiber	30 μ	15 μ
Electron microscopy a) thin section studies		
Presynaptic complex		Degeneration of presynaptic complex is gradual and the following events take place during 24 h after denervation:
	Schwann cells lie above the nerve terminal. The nerve terminal is pear-shaped and abuts the indented sarcolemma.	The processes of Schwann cells invade and surround the terminal. The nerve terminal is fragmented.
	Synaptic vesicles are randomly dispersed.	The synaptic vesicles appear clumped and decreased in number.
		Mitochondria are swollen.

TABLE I/p.2

FEATURES INVESTIGATED	CONTROL	DENERVATED
Presynaptic complex (continued)		The Schwann cells disintegrate and engulf the nerve terminal. By 24 h the nerve terminal disappears and only the Schwann cells remain in the region previously occupied by the nerve terminal.
Muscle fiber Post-synaptic complex		
Post-synaptic sarcolemma	Deeply indented into bulb-like structures in which the nerve terminal rests.	After denervation the bulb-like formation of sarcolemma is lost and it becomes straight.
	Synaptic folds reach deep into the sarcoplasm (5000-6000 A^0)	Some of the folds are retained as long as 2 months after denervation, but they appear reduced in number and depth.
Microfilaments	Long microfilaments are seen in contact with the post-synaptic sarcolemma.	Apparently the distribution pattern undergoes alteration and the microfilaments appear shorter in sections, though they are still in contact with post-synaptic sarcolemma.
	Bundles of microfilaments are never noticed.	Bundles of microfilaments are present.
Lysosomes	Lysosomes are rare if present at all.	Lysosomes in the form of multivesicular bodies are frequent in the soleplasm.
Membrane whorls	Membrane whorls are not found.	Membrane whorls are present.

TABLE I/p.3

FEATURES INVESTIGATED	CONTROL	DENERVATED
Non-synaptic complex		
Myofilaments	Reach almost up to the sarcolemma.	2 days after denervation, distortion of myofilaments occurs in peripheral sarcoplasm.
	In longitudinal sections there is always a parallel arrangement of myofilaments.	2 months after denervation, transversely as well as longitudinally organized myofilaments are seen in longitudinal sections. It appears that the arrangement of myofilaments is disorganized.
	Z-band is 400-500 A^{o} wide.	Remains 400-500 A^{o} wide but becomes wavy, fragmented and disoriented.
Lysosomes	Never found.	Markedly increased.
Vesiculation of the sarcoplasm	Never found.	Conspicuous.
Ribosomes and rough endoplasmic reticulum	Scanty in peripheral sarcoplasm except around nuclear region.	Marked increase in these organelles is seen 8-15 days after denervation.
Membrane whorls	Never found.	Abundant.
Lipid globules	Rarely noticed.	Common.
Mitochondria	Run in small rows in the interfibrillar sarcoplasm or are arranged in pairs opposite I-band.	8-15 days after denervation, paired arrangement of mitochondria is lost in many areas. 2 months after denervation, aggregates of mitochondria can be seen.

TABLE I/p.4

FEATURES INVESTIGATED	CONTROL	DENERVATED
b) Acetylcholin-esterase (AchE) activity	Uniformly distributed in synaptic clefts.	2 weeks after denervation, the enzyme activity is either absent or non-uniformly distributed in synaptic clefts. This is suggested to be due to loss of AchE (of 16s molecular form) from synaptic clefts.
c) Freeze-fracture		
Non-junctional sarcolemma		15 days after denervation, following changes are seen:
P-face (convex fracture)	80 A^0 particles are predominant.	80 A^0 particles are markedly decreased.
	Very few large particles (150-180 A^0) are present.	Increase in number of large particles (150-180 A^0).
	Packing density of particles (80 A^0) is 2000/μ^2.	Packing density of particles (mostly of 150-180 A^0 diameter) varies from 400-1000/μ^2
	The particles are always randomly distributed.	The distribution of these large particles varies over the P-face. The large particles are hypothesized to be representative of Ach receptor.

TABLE II. Comparison of junctional and extrajunctional Ach receptors

Features investigated	Junctional receptors	Extrajunctional receptors	References
1. α-Bungarotoxin binding sites in innervated muscle	$1.3 \times 10^4/\mu^2$	$5/\mu^2$	Hartzell and Fambrough (1972) Fambrough and Hartzell (1972)
2. α-Bungarotoxin binding sites in denervated muscle	Persists even 10 days after denervation. 60% loss occurs 4-5 weeks later	Increase in number to $1695/\mu^2$	Frank *et al.* (1975)
3. Receptor degradation as judged by loss of ^{125}I α-BGT binding to purified preparation	The purified receptor preparation from innervated muscle retains 65% of the radioactivity 5 days after binding	In the receptor preparation from denervated muscle, no toxin can be detected 5 days later	Frank *et al.* (1975)
4. Direct electrical stimulation of denervated muscle	Loss of receptors is not prevented	Reduces the number of extrajunctional receptors	Frank *et al.* (1975) Lomo and Westgaard (1975)

TABLE II/p.2

Features investigated	Junctional receptors	Extrajunctional receptors	References
5. Interaction with tubocurarine	Decreases the reaction of receptor with α-BGT. However, at low concentration (5×10^{-8}M) the junctional receptors are more effective in inhibiting the reaction of receptors with α-BGT	Same At low concentration it is less effective in inhibiting the reaction of receptors with α-BGT	Brockes *et al.* (1975)
6. Dissociation constant of tubocurarine-receptor complex	4.5×10^{-8}M	5.5×10^{-7}M	Brockes *et al.* (1975)
7. Isoelectric focusing of α-BGT-receptor complex	Major peak at 5.1	5.3	Brockes *et al.* (1975)
8. Sedimentation constant	9s	9s	Brockes *et al.* (1975)

TABLE II/p.3

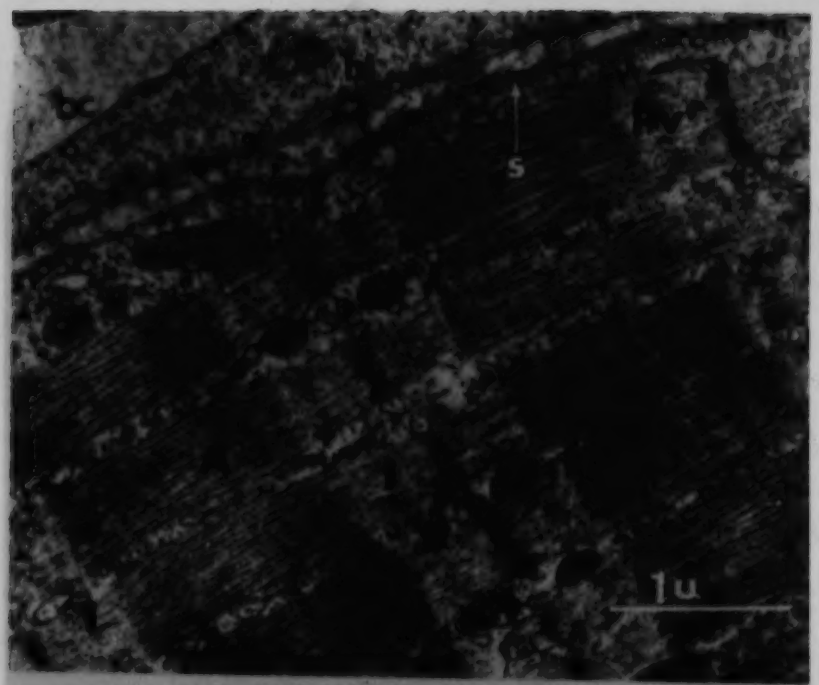
Features investigated	Junctional receptors	Extrajunctional receptors	References
9. Reaction with Concanavelin A (ConA)	Binds to ConA and therefore glycoprotein in nature	Same	Brockes *et al.* (1975)
10. Reaction with serum raised against the eel Ach receptors	Precipitation curves for both junctional as well as extrajunctional receptors are the same		Brockes *et al.* (1975)
11. Freeze-fracture studies	The particles (150 A^{O}) representing Ach receptors are present on the P-face		Rash and Ellisman (1974)

PLATE 1

Plates 1-23. Control muscle.

Figure 1. Longitudinal section of lumbrical muscle cell showing the outer basement lamina (bl), invaginations of the sarcolemm Sarcolemma (pv). The labels A, I and bc refer to A-band, I-band and blood capillary respectively.

Figure 2. Part of the muscle fiber stained with ruthenium red Showing association of collagen fiber (c) with basement lamina (bl). Ruthenium red positive material is seen around collagen fibers, pinocytotic vesicles (pv) and in the basement lamina (bl).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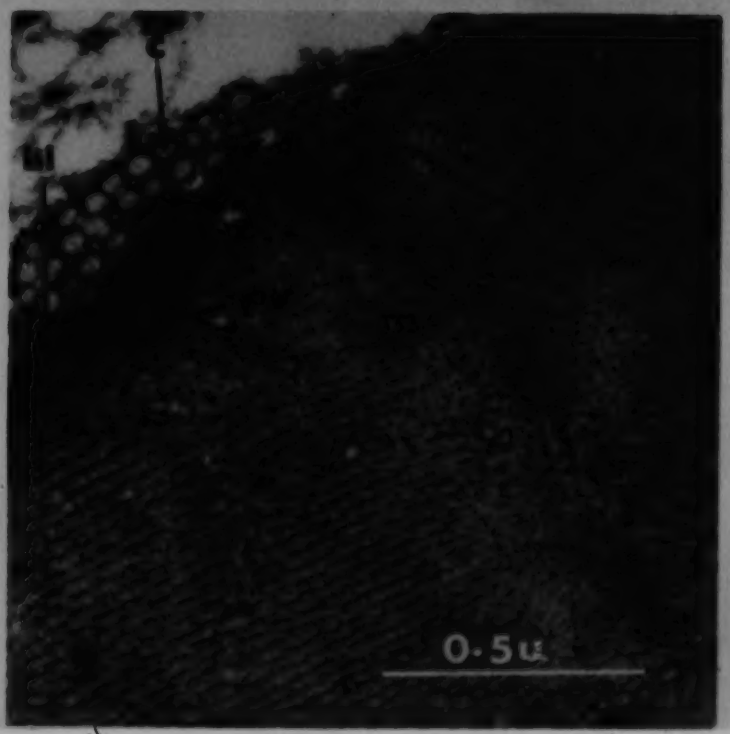
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PLATE 2

Figure 3. Part of the muscle cell showing nucleus (N) in the peripheral region and abundant ribosomes in the perinuclear sarcoplasm. The letters A, I and Z indicate the bands of the myofibril. M-line is indicated by the arrowhead. The interfibrillar sarcoplasm contains sarcoplasmic reticulum (sr), transverse tubules (tt) and mitochondria (m) which are in pairs (arrows) opposite I-band.

Figure 4. Part of the muscle cell showing small rows of mitochondria (m) in the interfibrillar sarcoplasm. Peripheral sarcoplasm shows conglomerates of mitochondria in the vicinity of blood capillary (bc).

1u

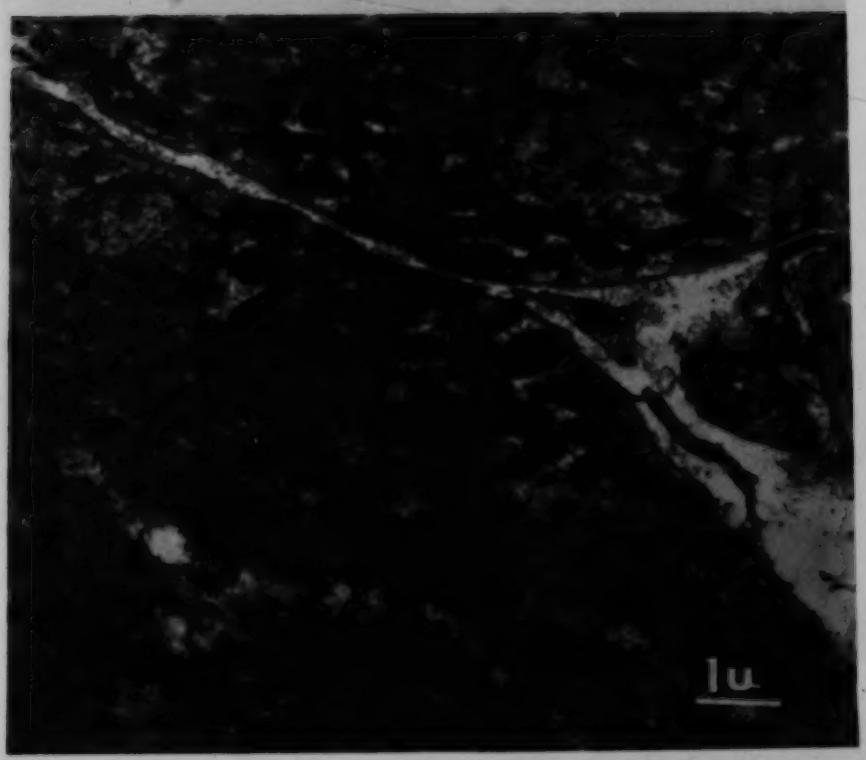

1u

PLATE 3

Figure 5. Sarcoplasm of the muscle fiber showing the triads (tr) at the A-I junction. Running between the triads are the elements of sarcoplasmic reticulum (arrowhead) and glycogen granules.

Figure 6. The structure of the triad at higher magnification showing central transverse tubule (tt) and terminal cisternae (tc) of the sarcoplasmic reticulum. Electron dense lines (shown by the arrowhead) are seen between the membranes of t-tubule and terminal cisternae.

1u

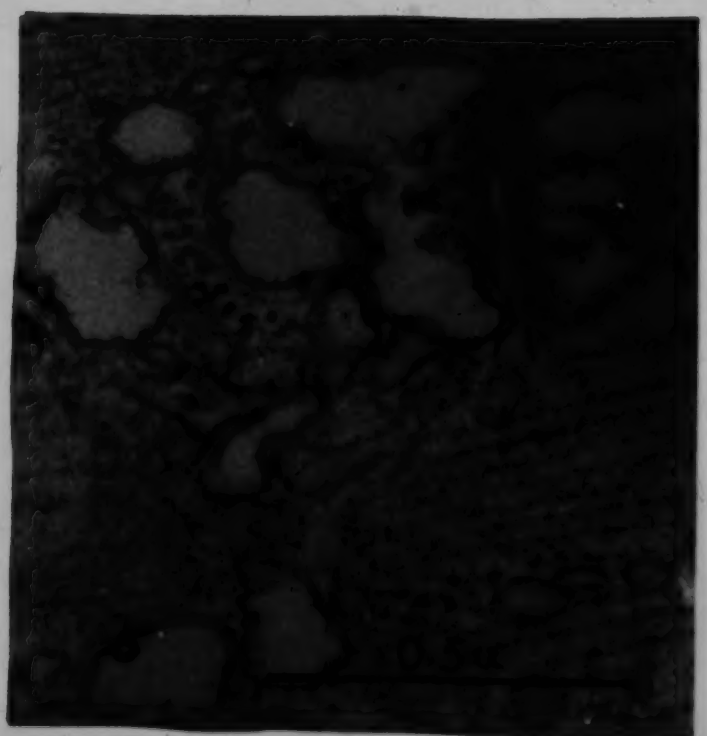


PLATE 4

Figure 7. Cross section through a nerve fiber showing Schwann cell (sc) and myelinated axon (max). The connective tissue layer (pn) is seen surrounding the axon. The neurofilaments are seen in the axoplasm.(axp).

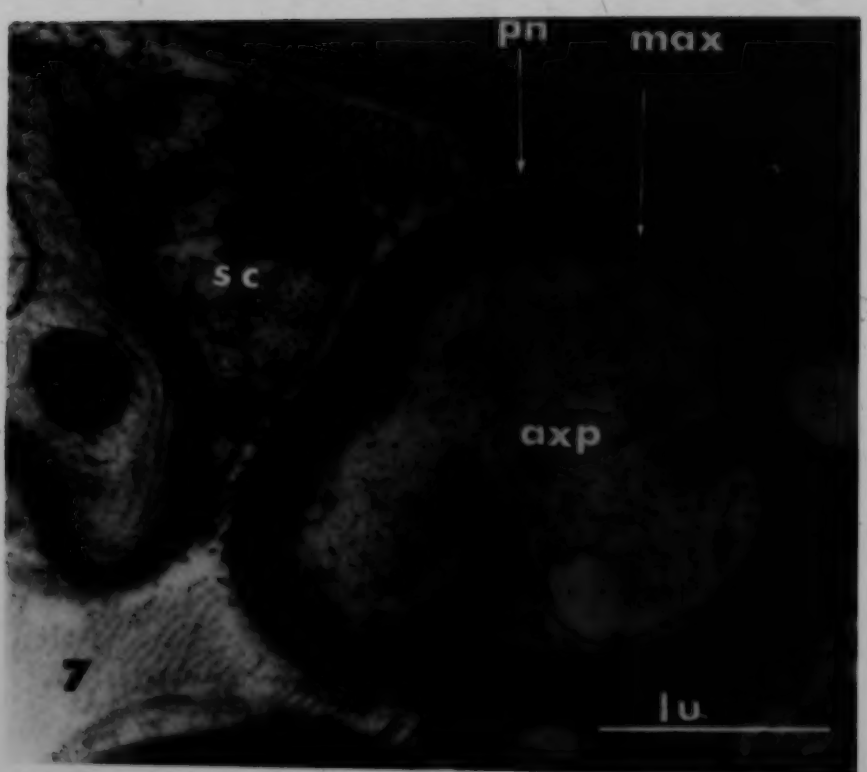

pn
max
sc
axp
7
lu

PLATE 5

Figure 8. Section through neuromuscular junction showing nerve terminal (NT) resting in the bulb like invagination of the sarcolemma. The nerve terminal contains synaptic vesicles (sv) 500-600 Å in diameter and mitochondria (m). On the postsynaptic side the sarcolemma is extensively invaginated forming several postsynaptic folds (psf). The basement lamina (bl) is seen to follow the invaginations of sarcolemma.

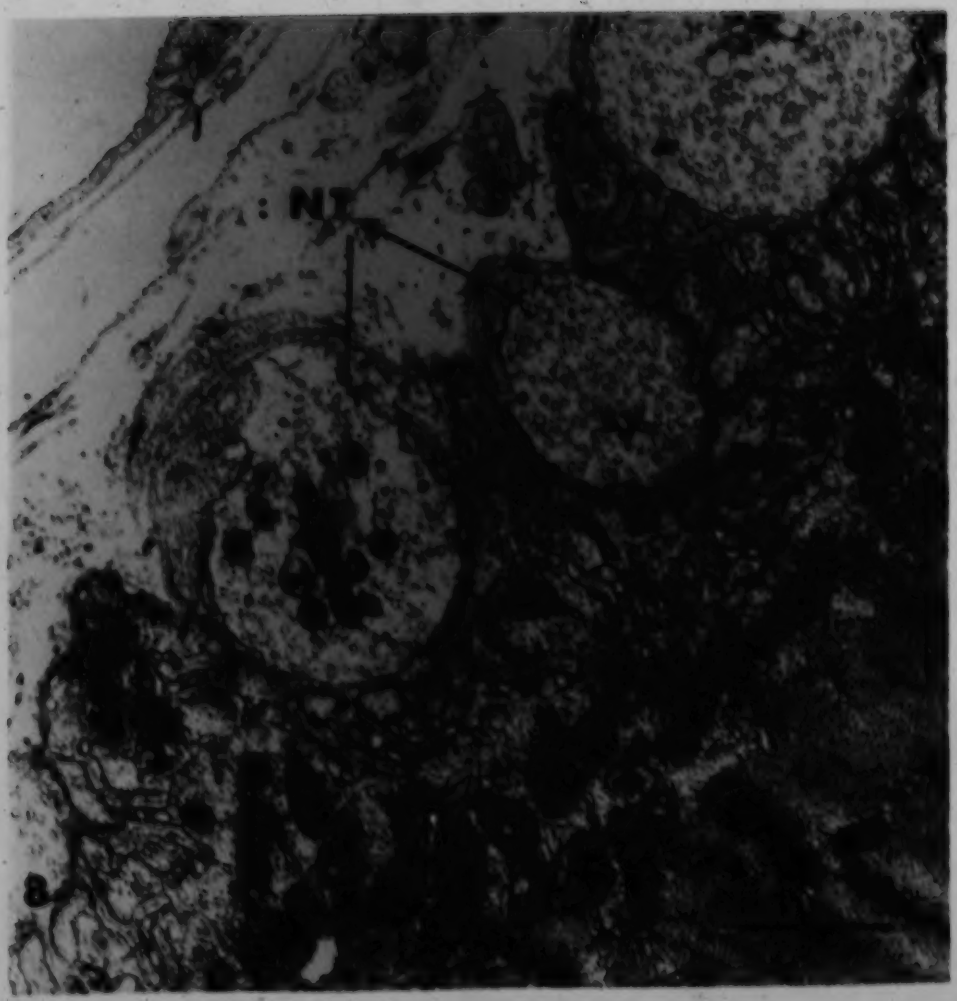

NT

PLATE 6

Figure 9. Synaptic region showing the nerve terminal (NT), basement lamina (arrowheads) in the synaptic cleft (sc) and soleplasm (sp) containing several ribosomes and microfilaments (mif). Some of these microfilaments (arrows) are seen coming in contact with the sarcolemma.

Figure 10. Portion of the soleplasm (sp) showing fibrous material (black arrowheads) associated with the sarcolemma. The basement lamina is indicated by clear arrowheads.

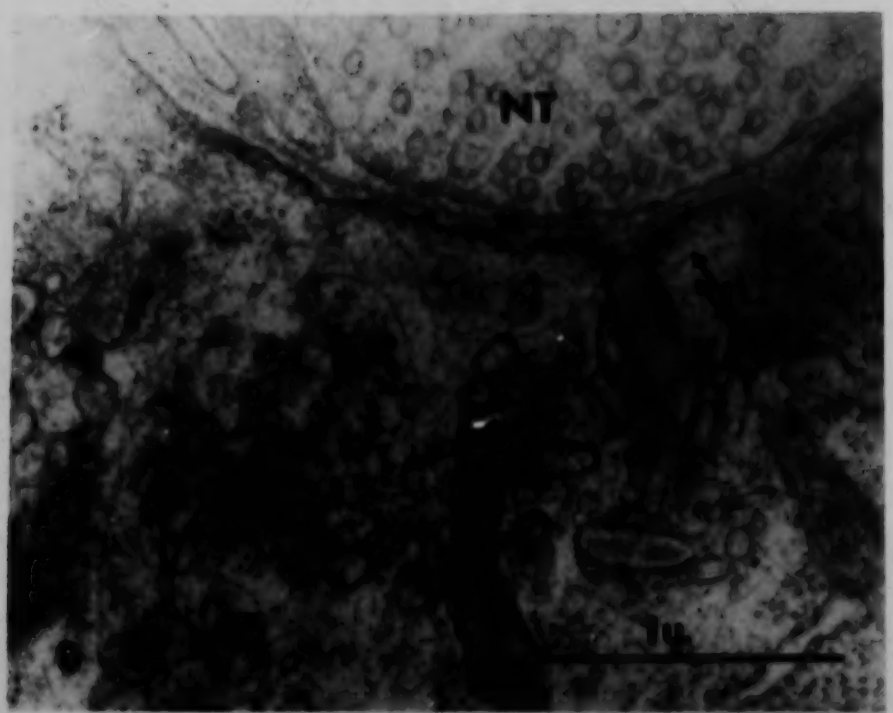

NT
1μ

PLATE 7

Plates 7-9. Histochemical localization of AchE in control muscle.

Figure 11. Section through motor end plate showing uniform distribution of AchE activity in the synaptic cleft(sc). Nerve terminal (NT) does not show any enzyme localization.

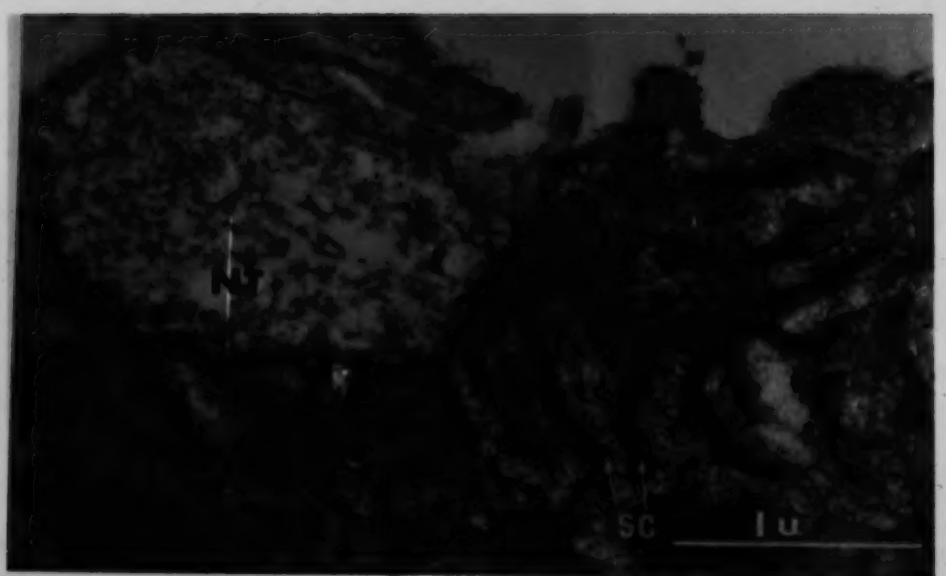
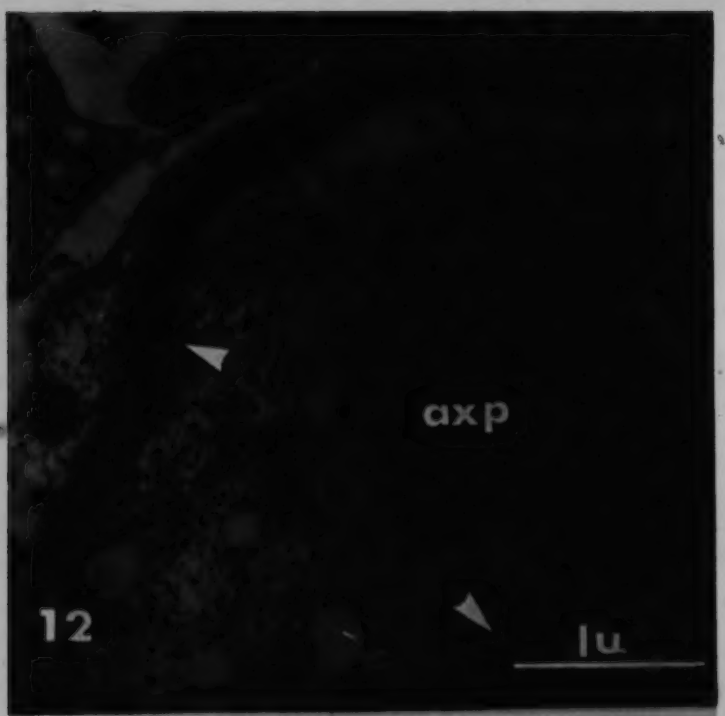

SC
1u

PLATE 8

Figure 12. Sectin through myelinated axon showing non-uniform AchE activity in the axonal membrane (arrowheads). The abbreviation axp refers to axoplasm.

Figure 13. Part of the muscle cell showing the AchE reaction product associated with the sarcoplasmic reticulum (arrows).


axp
12
lu

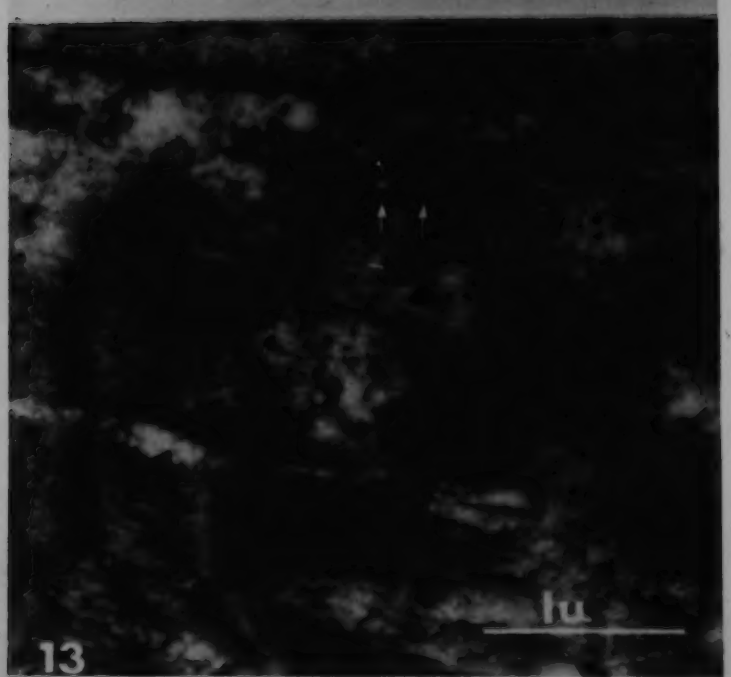

lu
13

PLATE 9

Figure 14. Section of a control muscle incubated in medium containing eserine sulfate which is an inhibitor of AchE. No reaction product is apparent in synaptic cleft (arrowhead) or in the nerve terminal (NT).

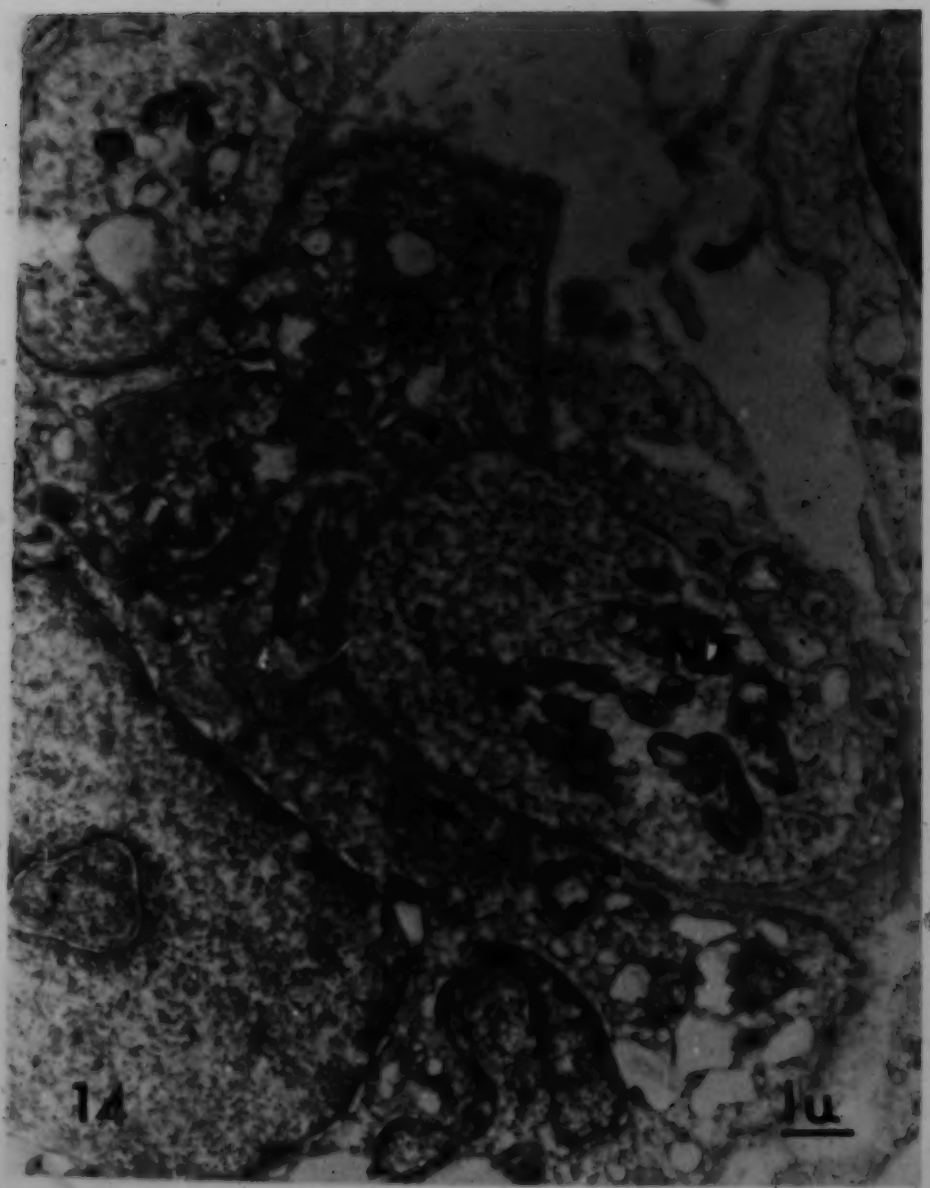

14
1u

PLATE 10

Figure 15. Diagramme showing the terms used to describe the fracture faces of the plasma membrane. The dotted line represents the line of fracture. The E-face (E) is that half of the membrane closer to the extracellular space. The P-face (P) isthat half of the membrane adjacent to the cytoplasm. S indicates the hydrophilic surface that can be exposed by etching.

Figure 16. Diagrammatic representation of plasma membrane showing the asymmetric arrangement of proteins (pr) in the lipid bilayer (li). There are more particles attached to the cytoplasmic half (P) than to the extracellular half (E) of the membrane. Therefore upon fracture, the P-face reveals more particles than the E-face.

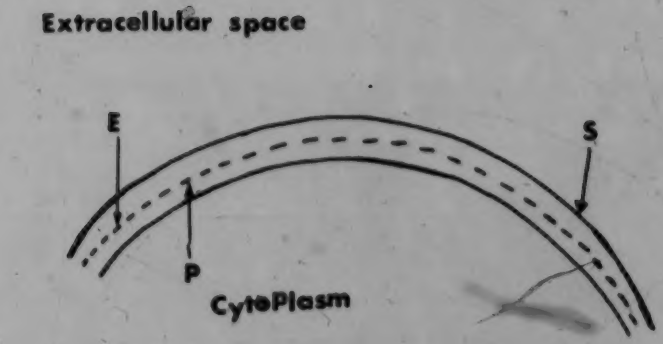


Figure 15

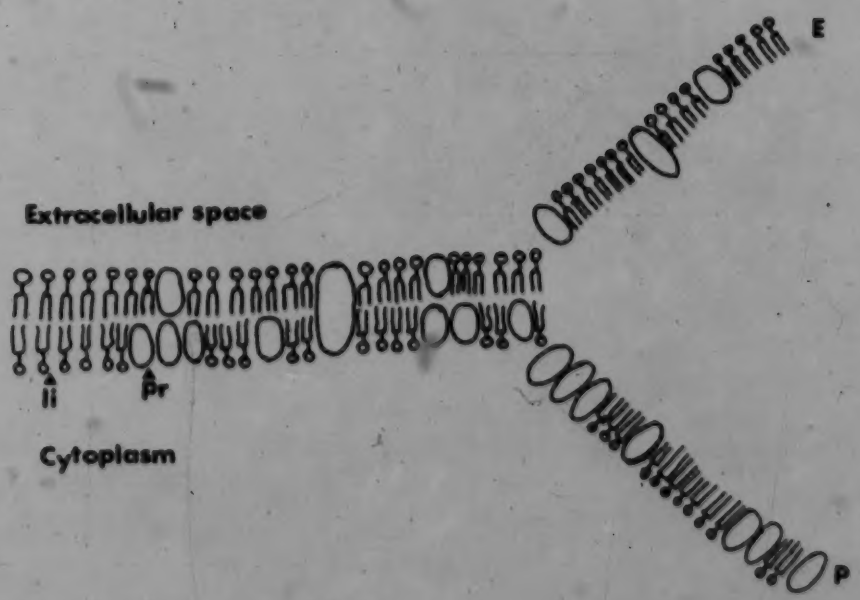


Figure 16

PLATE 11

PLATES 11-13. Fracture faces of non-synaptic sarcolemma from control muscle.
Large arrows indicate the direction of shadowing in these and all subsequent freeze-fracture micrographs.

Figure 17. P-face of non-synaptic sarcolemma showing depessions and random distribution of 80 A^{o} particles. The muscle cytoplasm (M) is seen below the fracture face.

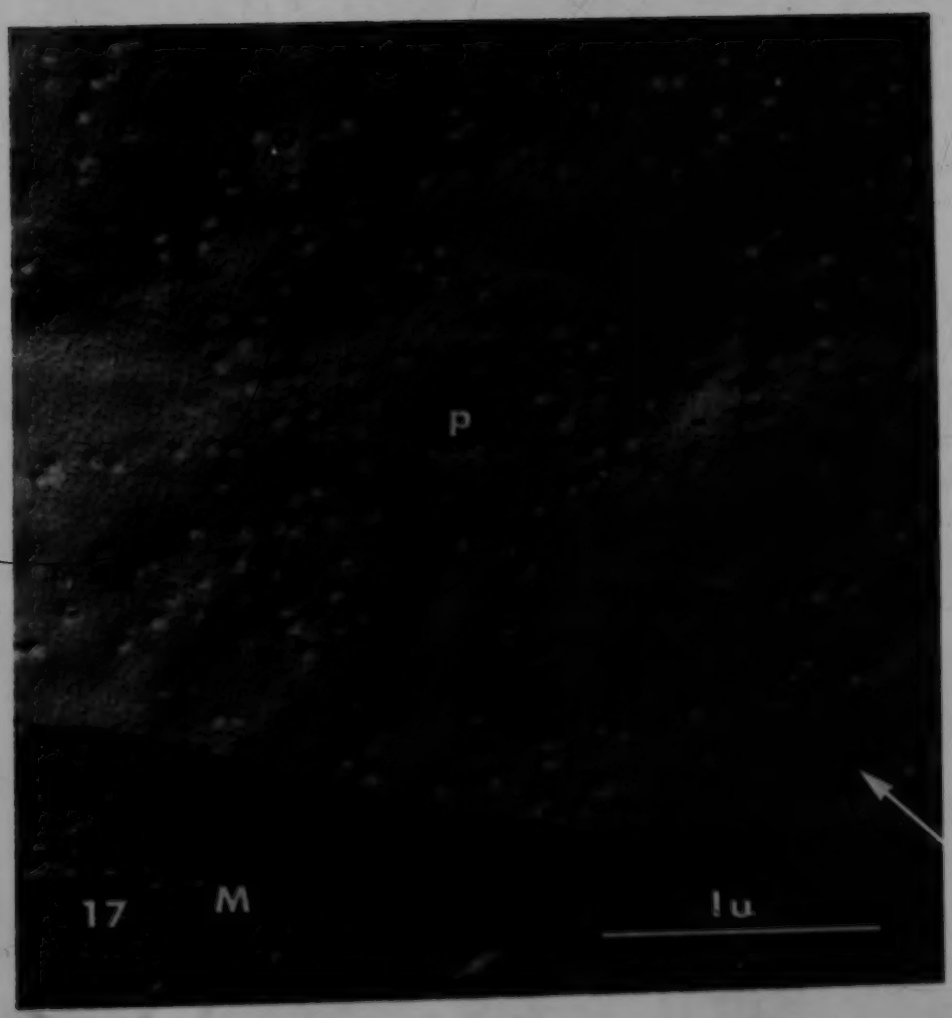
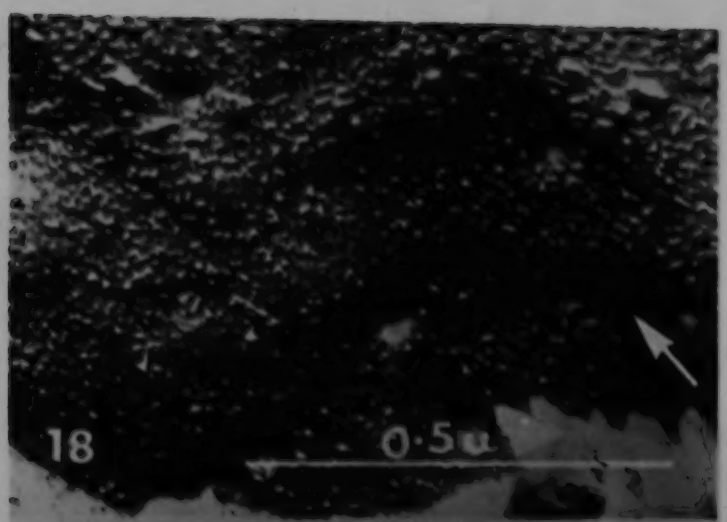

p
17
M
1u

PLATE 12

Figure 18. P-face of non-synaptic sarcolemma showing square arrays (arrowheads) of particles.

Figure 19. P-face of non-synaptic sarcolemma showing group of large particles (arrowhead). These particles are 150-180 A^{o} in diameter and are only occasionally seen on P-face.


18
0·5u

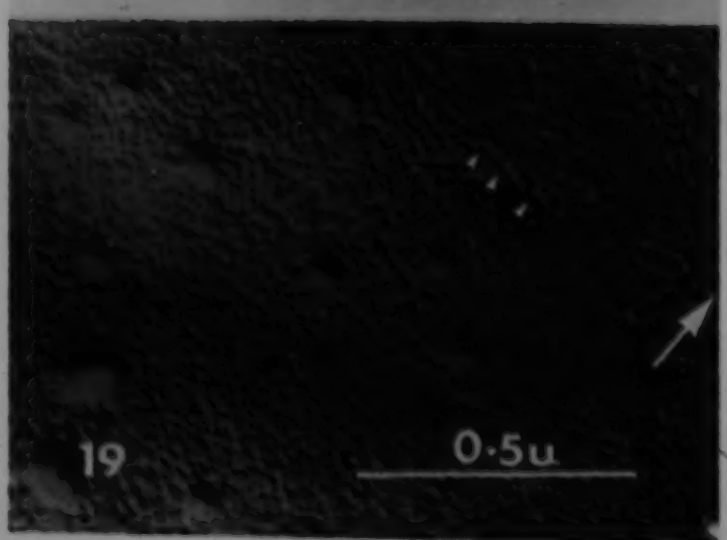

19
0·5u

PLATE 13

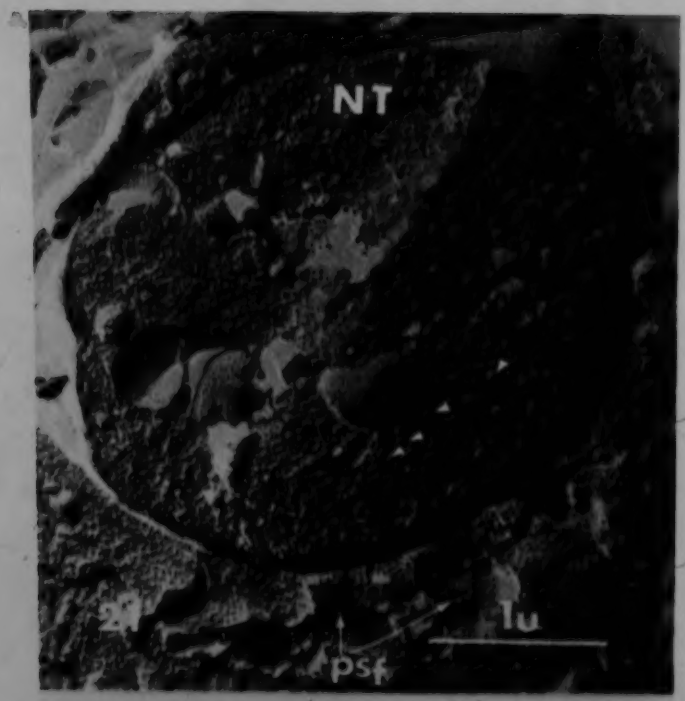
Figure 20. The E-face of the non-synaptic sarcolemma showing protuberances and few 80 A particles.

E
20
1u

PLATE 14

Figure 21. Cross fracture through synapse showing nerve terminal(NT) containing synaptic vesicles (arrowheads). On the postsynaptic side few fracture faces of synaptic folds (psf) can be seen.

Figure 22. Thin section electron micrograph of synaptic region corresponding to the freeze-fracture replica shown in figure 21. This shows the nerve terminal (NT) containing synaptic vesicles (arrowheads).The abbreviation psfrefers to the post-synaptic folds.


NT
22
psf
lu

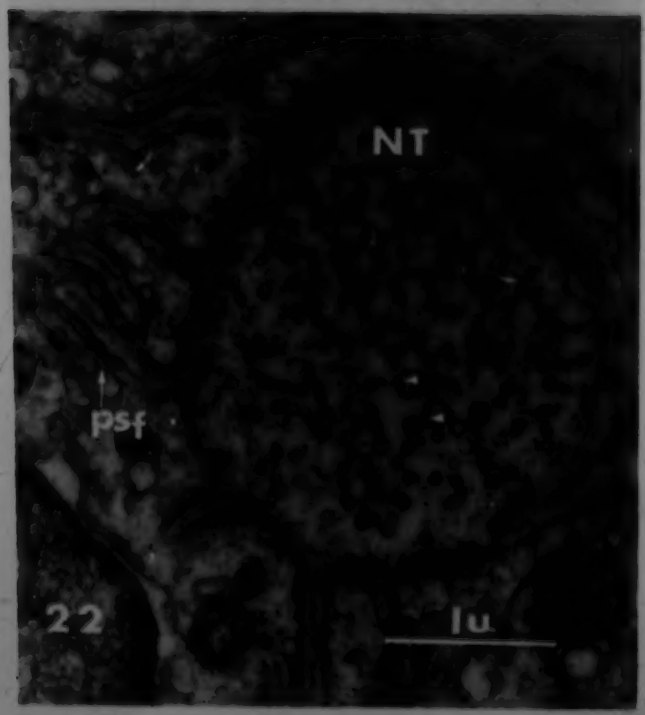

NT
psf
22
lu

PLATE 15

Figure 23. Freeze-fracture electron micrograph of a synaptic region showing the P-face of the nerve terminal (PNT). Note the characteristic double rows of 100 A particles (indicated by circles). Particles of 150-180 A (arrowhead) are present on the P-face (P) of the post-synapyic folds. The E-face (E) shows fewer particles.

PNT
P
E
P
E
Psf
23
lu

PLATE 16

Figure 24. Changes in the weight of the denervated muscle with time expressed as a percentage of the weight of control muscle. Following denervation, the weight of the denervated muscle increases up to 4 days and then begins to decrease. By 15 days, the denervated muscles weigh 75% of the controls

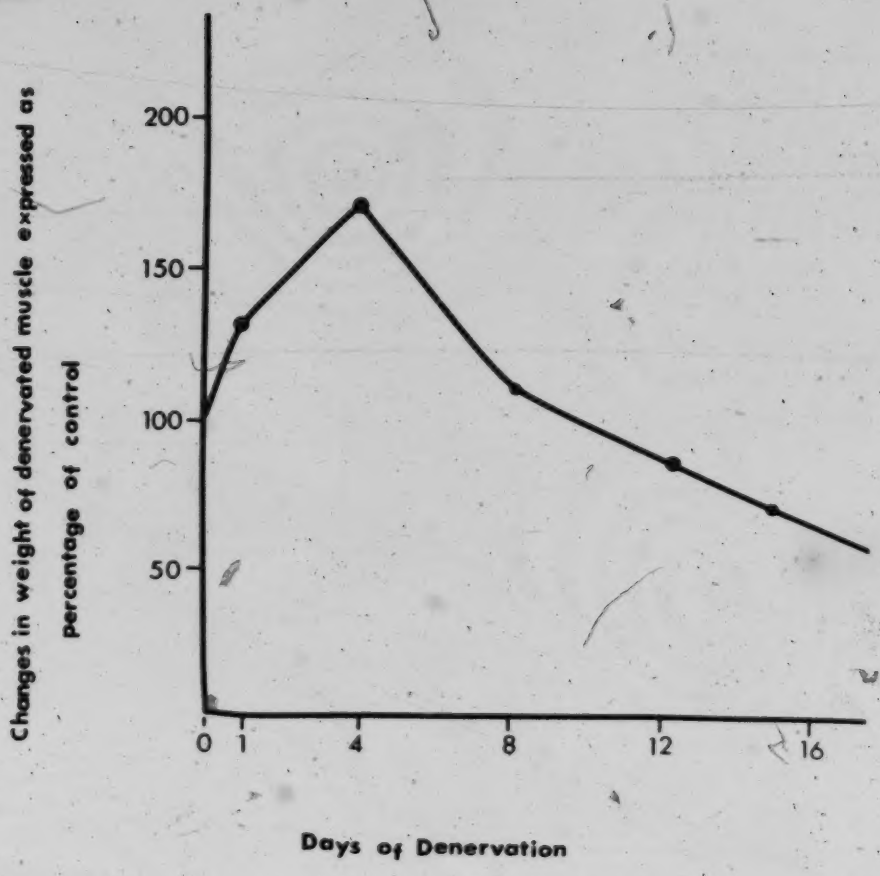

Changes in weight of denervated muscle expressed as percentage of control
200
150
100
50
0
1
4
8
12
16
Days of Denervation

Fig: 24

PLATE 17

Structure of nerve terminal 6h after denervation.

Figure 25. A part of the nerve terminal shows the presence of membrane whorl in the axoplasm. (indicated by the arrow).

Figure 26. The axoplasm of the nerve terminal shows clumped synaptic vesicles (arrowheads).

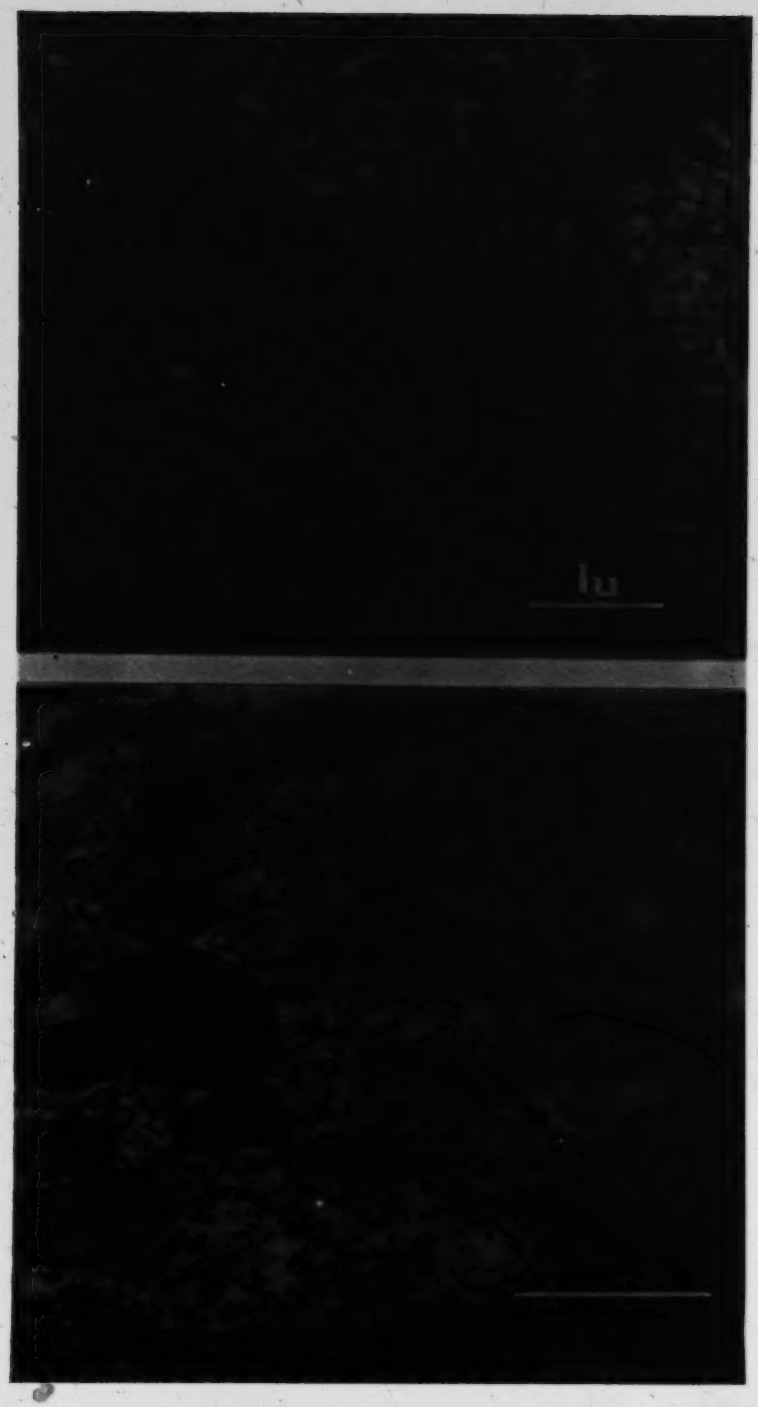
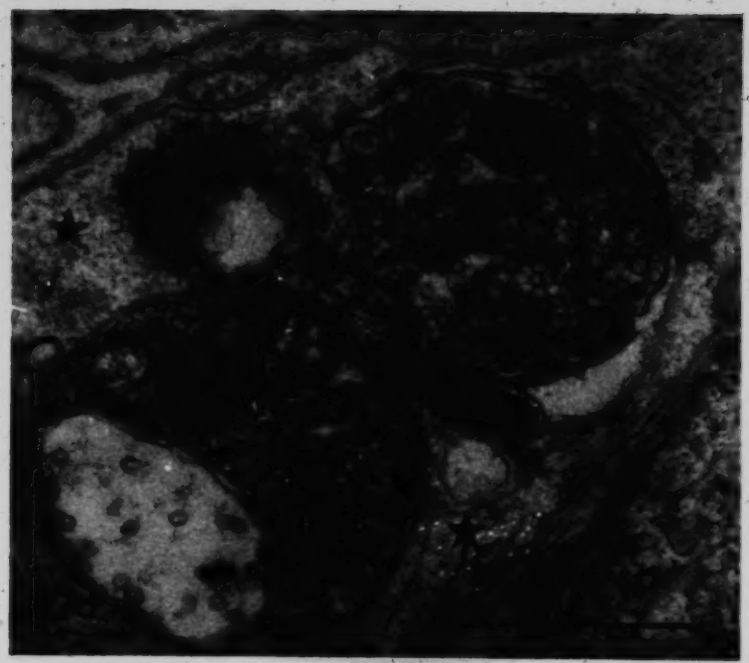

1u

PLATE 18

Disintegration of the nerve terminal 12h after denervation.

Figure 27a. The cytoplasm of the Schwann cell (star) is seen surrounding the nerve terminal (NT). Within the nerve terminal clumped synaptic vesicles are seen.synaptic cleft is indicated by the arrowhead.

Figure 27b. Low-power micrograph of neuromuscular junction showing the fragmentation of the nerve terminal (NT) by the Schwann cell (Sc). The arrrowhead indicates the clumped synaptic vesicles.



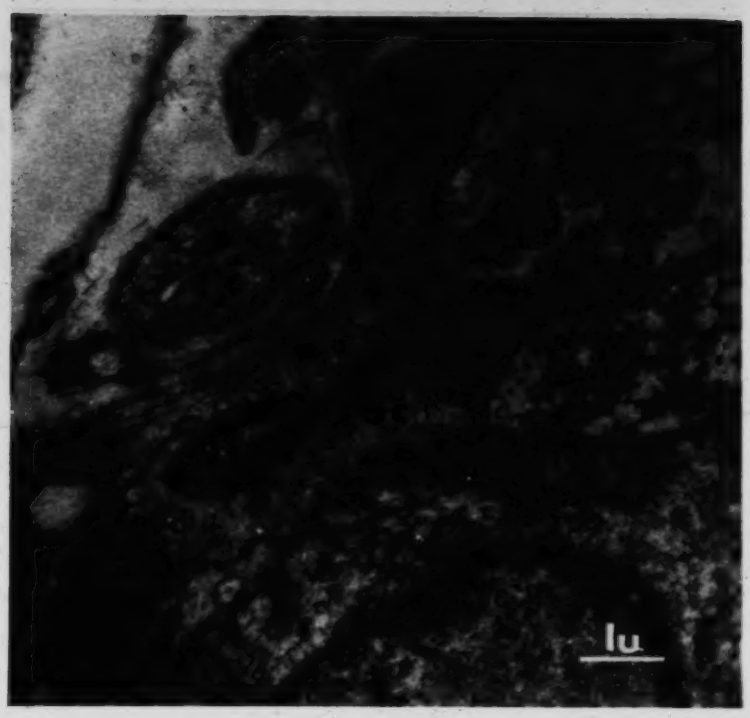

1u

PLATE 19

Changes in the nerve terminal 12h after denervation.

Figure 28. The nerve terminal (NT) showing decrease in number of synaptic vesicles and swollen mitochondria (m).

Figure 29. Part of the synaptic region where nerve terminal (NT)is seen enclosed in processes of Schwann cell (Sc). Axoplasm shows decreased number of synaptic vesicles. A memranous vesicle resembling synaptic vesicle(arrowhead) can be seen in the synaptic cleft.

PLATE 20

Figure 30a & b. Part of the synaptic complex 24h after denervation showing the replacement of nerve terminal by Schwann cell. The Schwann cell contains dense material (arrowhead) which probably is the phagocytised nerve treminal.

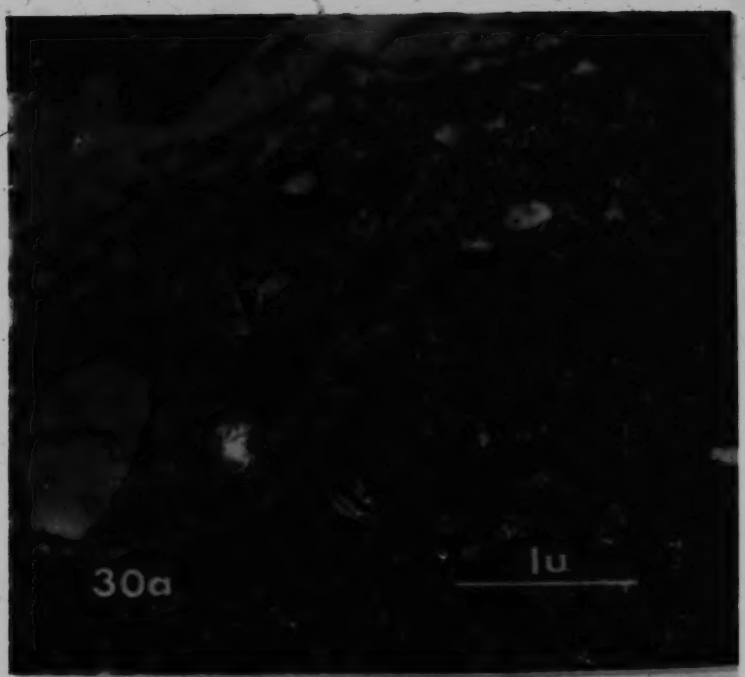
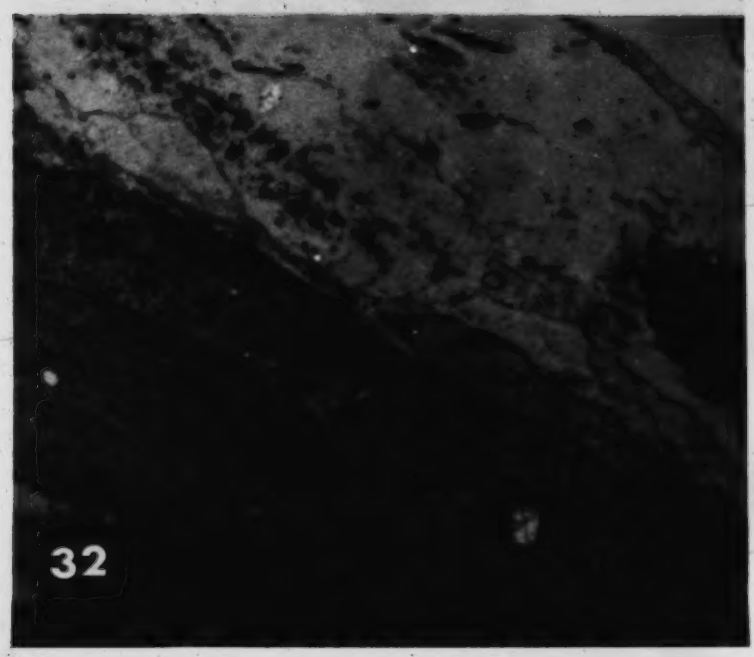

30a
1u

PLATE 21

Figure 31. The post-synaptic complex 2days after denervation showing the reduction in depth of synaptic folds (arrowhead).

Figure 32. The post-synaptic complex 15 days after denervation showing loss of folds (shown by arrowhead). Few synaptic folds are retained and are indicated by the arrows.

31



32

PLATE 22

Figure 33. The post-synaptic complex 4 days after denervation showing the microfilaments (arrowheads) in soleplasm (SP) which appear to be shorter in length than in control muscle (compare with figure 9).

1u

PLATE 23

Figure 34. Soleplasm (SP) 4 days after denervation showing lysosomes (arrows) and bundles of microfilaments (arrowheads).

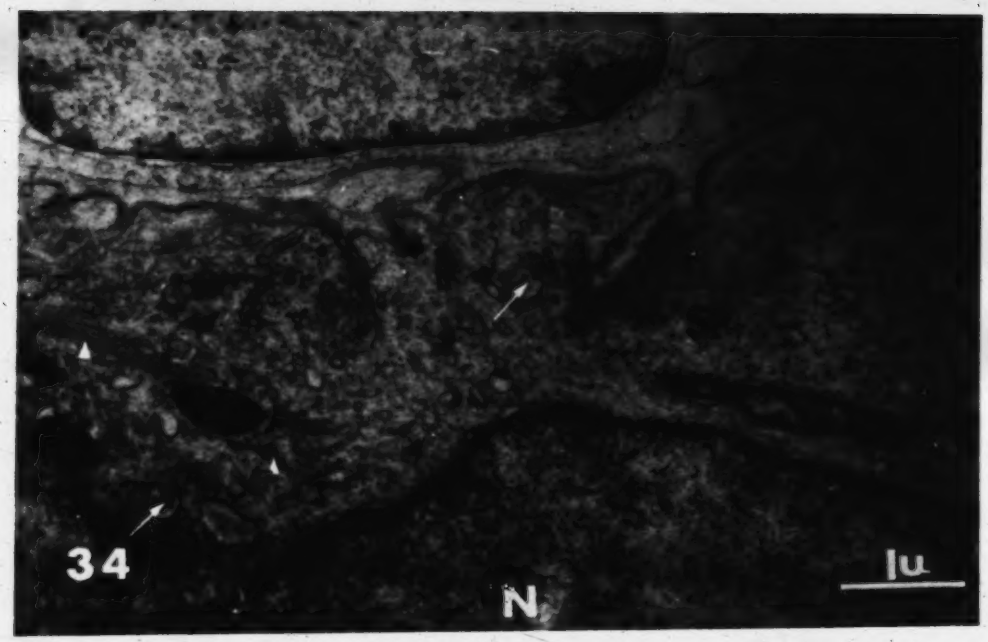

34
N
1u

PLATE 24

Figure 35. Muscle denervated for 15 days and stained with ruthenium red. The basement lamina is intensely stained with ruthenium red positive material

PLATE 25

Figure 36. Part of the muscle 4 days after denervation showing peripheral (P) disruption of myofilaments. Electron opaque region are the Z-bands (arrowhead).

Figure 37. Higher magnification of peripheral sarcoplasm showing disrupted myofilaments (arrowheads) and Z-band (Z). Lysosomes (ly) can be seen in the sarcoplasm.

PLATE 26

Figure 38. Longitudinal section of the muscle (denervated for 2 months) showing the orientation of myofilaments The Z-band (arrow) appear wavy. (arrowhead).

Figure 39. Longitudinal section of muscle (denervated for 2 months) showing transversely aligned myofilaments. (star). The Z-band appears fragmented. (arrowhead).

38

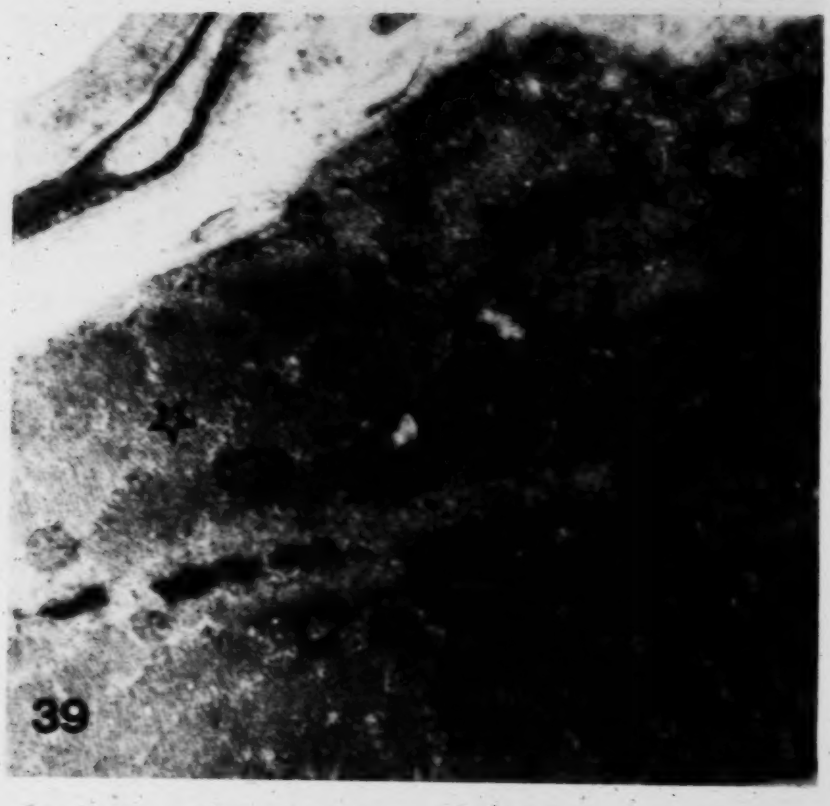
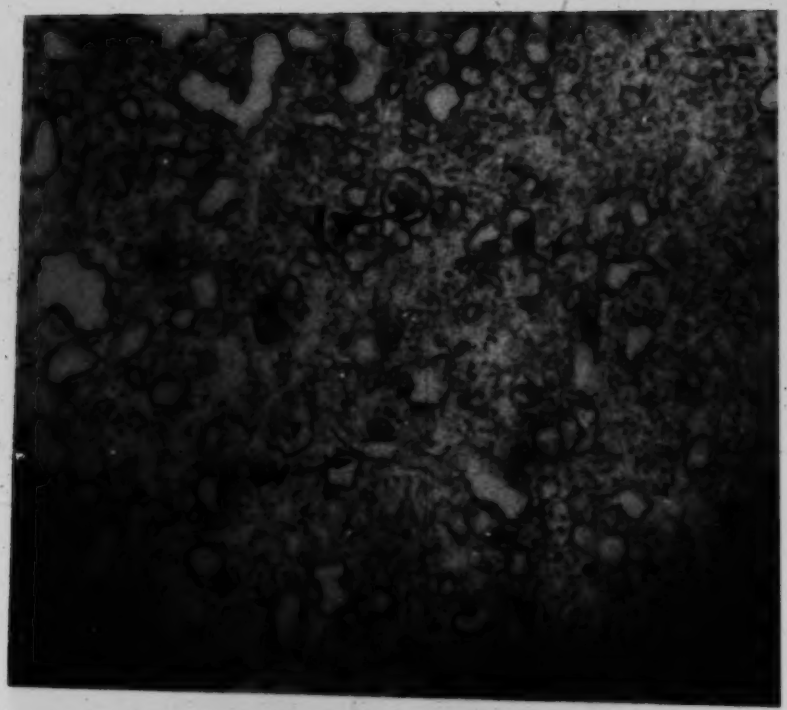

39

PLATE 27

Figure 40. Part of the sarcoplasm from muscle denervated for 2 days showing the presence of lysosomes (ly) and an increase in smooth vesicles of endoplasmic reticulum. (sv)

Figure 41. Membrane whorl in the peripheral sarcoplasm of muscle denervated for 2 days.



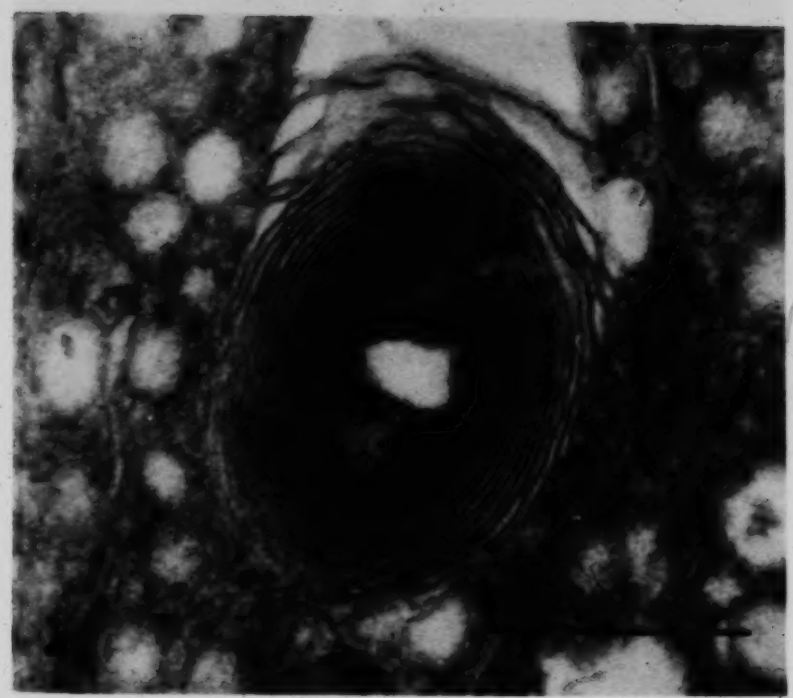


PLATE 28

Figure 42. 15 days denervated muscle showing the presence of lysosomes (arrowhead) and lipid in the interfibrillar sarcoplasm. A membrane whorl (mw) is opposite the I-band.

PLATE 29

Figure 43. Vacuolation of the sarcoplasm in muscle denervated for 4 days.

Figure 44. Higher magnification of the sarcoplasm showing the amorphous content in the interior of vacuoles.

PLATE 30

Figure 45. Section through muscle denervated for 4 days showing the vacuolated nucleoplasm.

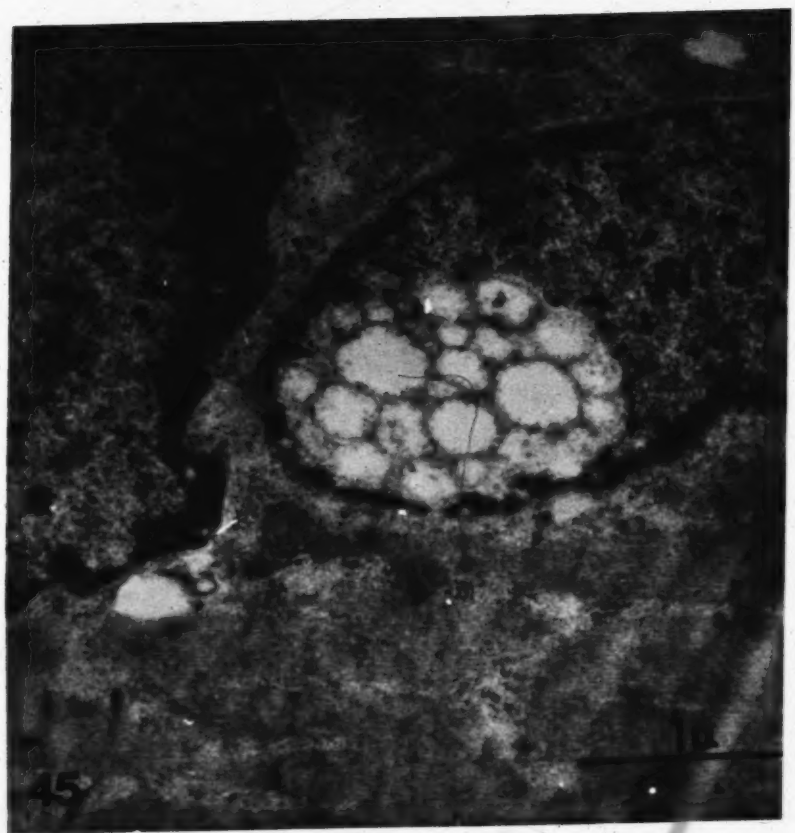

45

PLATE 31

Figure 46. The peripheral sarcoplasm (P) of control muscle to illustrate the infrequent occurrence of ribosomes.

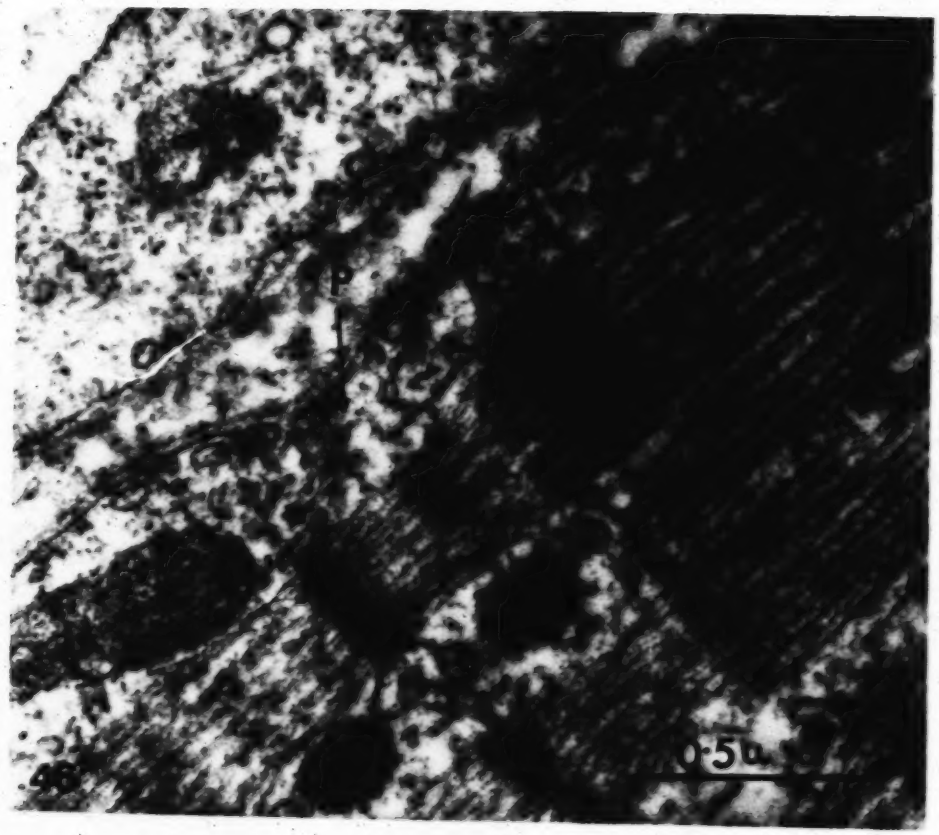

P
0·5μ

PLATE 32

Figure 47a. Peripheral sarcoplasm (P) of muscle denervated for 15 day days showing an increase in the number of electron opaque particles.

Figure 47b. The peripheral sarcoplasm (P) of muscle (denervated for 15 days) which is incubated with ribonuclease. There is parial loss of these particles which are free and the ones that are attached to the endoplasmic reticulum.

P
0.5u

P

PLATE 33

Figure 48. Sarcoplasm of muscle (denervated for 15days) showing the lipid globule opposite I-band. Mitochondrian (m) is seen in close association with the lipid (li).

Figure 49. Sarcoplasm of 2 month denervated muscle showing the presence of lipid globule (li).

1u

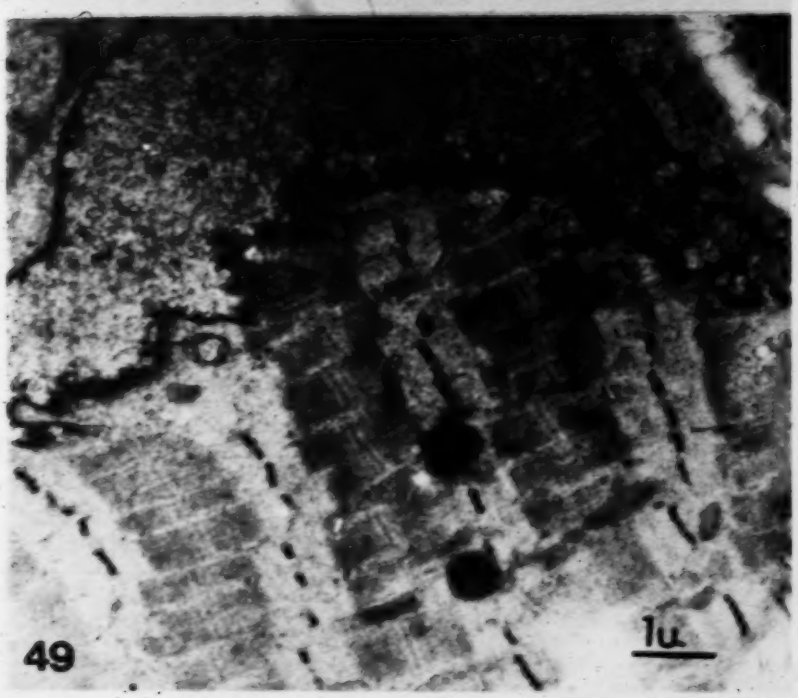

49
1u

PLATE 34

Figure 50. Section through the muscle (denervated for 2 months) showing the aggregates (arrowhead) of mitochondria in the sarcoplasm.

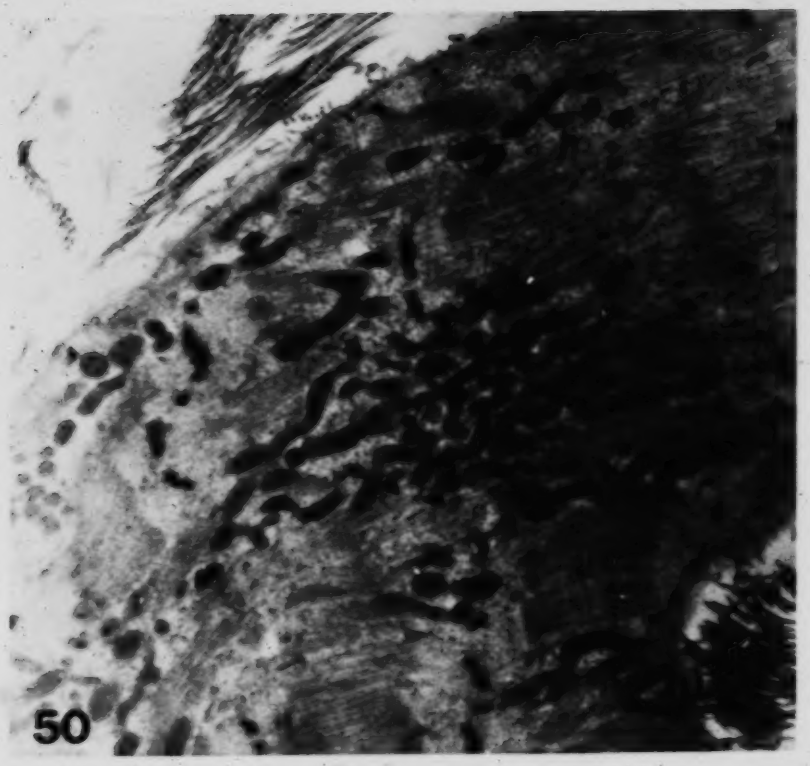

50

PLATE 35

Acetylcholinesterase activity of a muscle denervated for 15 days.

Figure 51. part of the post-synaptic region shows non-uniform distribution of enzyme activity.

Figure 52. Part of the muscle fiber showing post-synaptic clefts which are devoid of AchE activity.

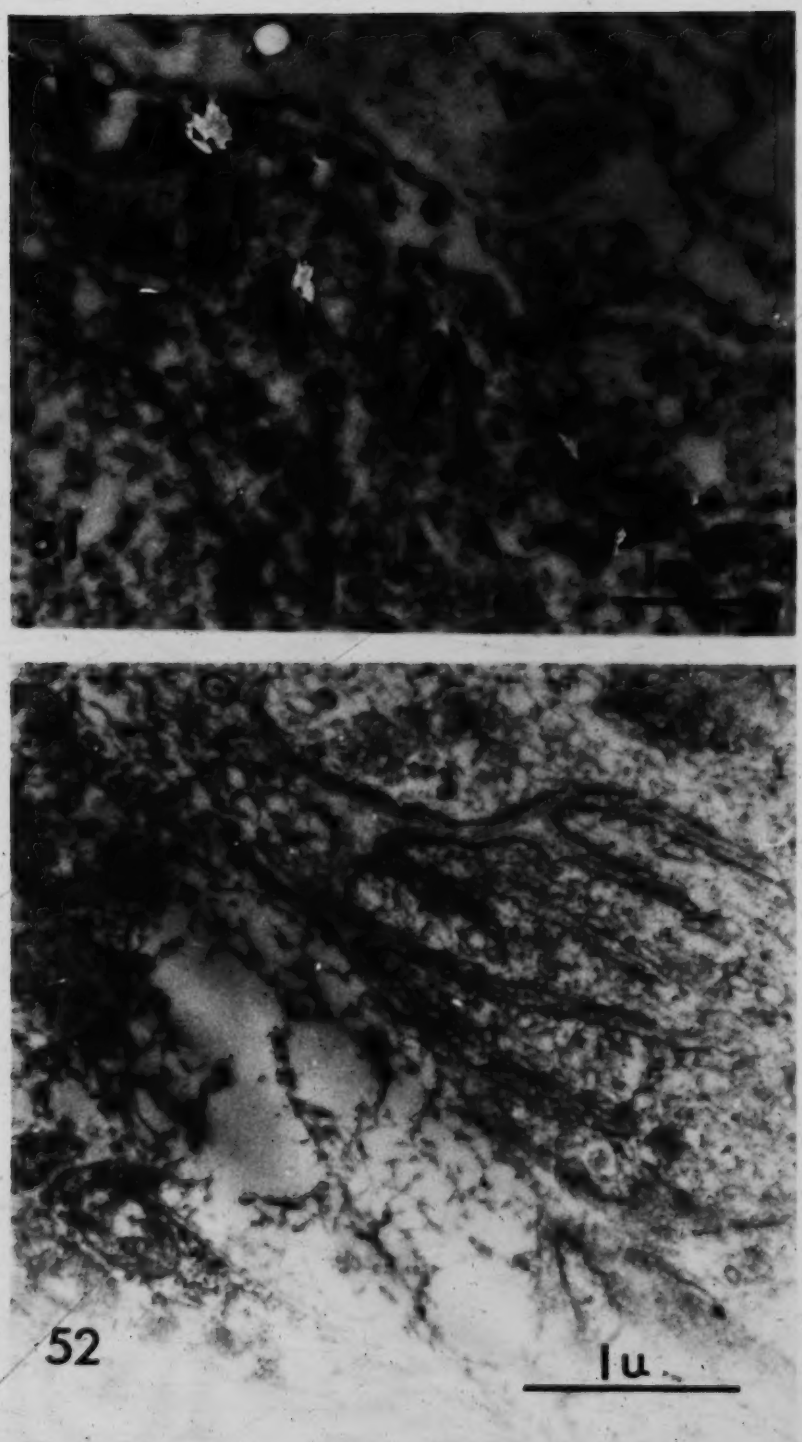

52
1u

PLATE 36

Figure 53. Inhibition of AchE activity in muscle denervated for 15 days

Figure 53. Part of the post-synaptic region of a muscle incubated in a medium containing eserine sulfate which is an inhibitor of AchE activity. The enzyme activity is absent in the synaptic clefts.

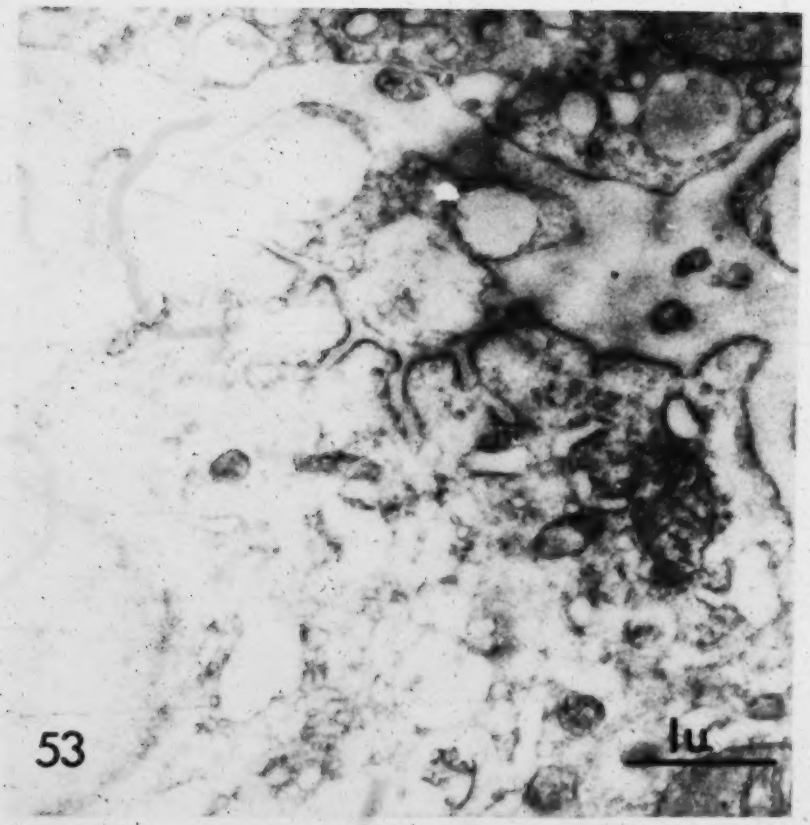

53
1μ

PLATE 37

Comparison of fractured faces of non-synaptic sarcolemma of control and muscle denervated for 15 days. (Large arrows indicate the direction of shadowing in these and all subsequent freeze-fracture micrographs.

Figure 54. P-face (p) of control muscle showing randomly distributed 80 A^{o} particles. The density of these particles is about 2000 particles/u^2.

Figure 55. P-face of denervated muscle showing the predominance of large particles (150-180 A^{o}) and a decrease in the number of small particles (80A^{o}). The packing density of these particles is about 400-1000/u^2 on P-face (P).

P
54
0·5u

E
P
55
0·5u

PLATE 38

Fractured faces of non-synaptic sarcolemma of muscle denervated for 15 days.

Figure 56. Part of P-face (P) showing many aggregates consisting of 4-15 large particles (150-180A°).

Figure 57. P-face showing large aggregates containing 30-50 particles (150-180A°).

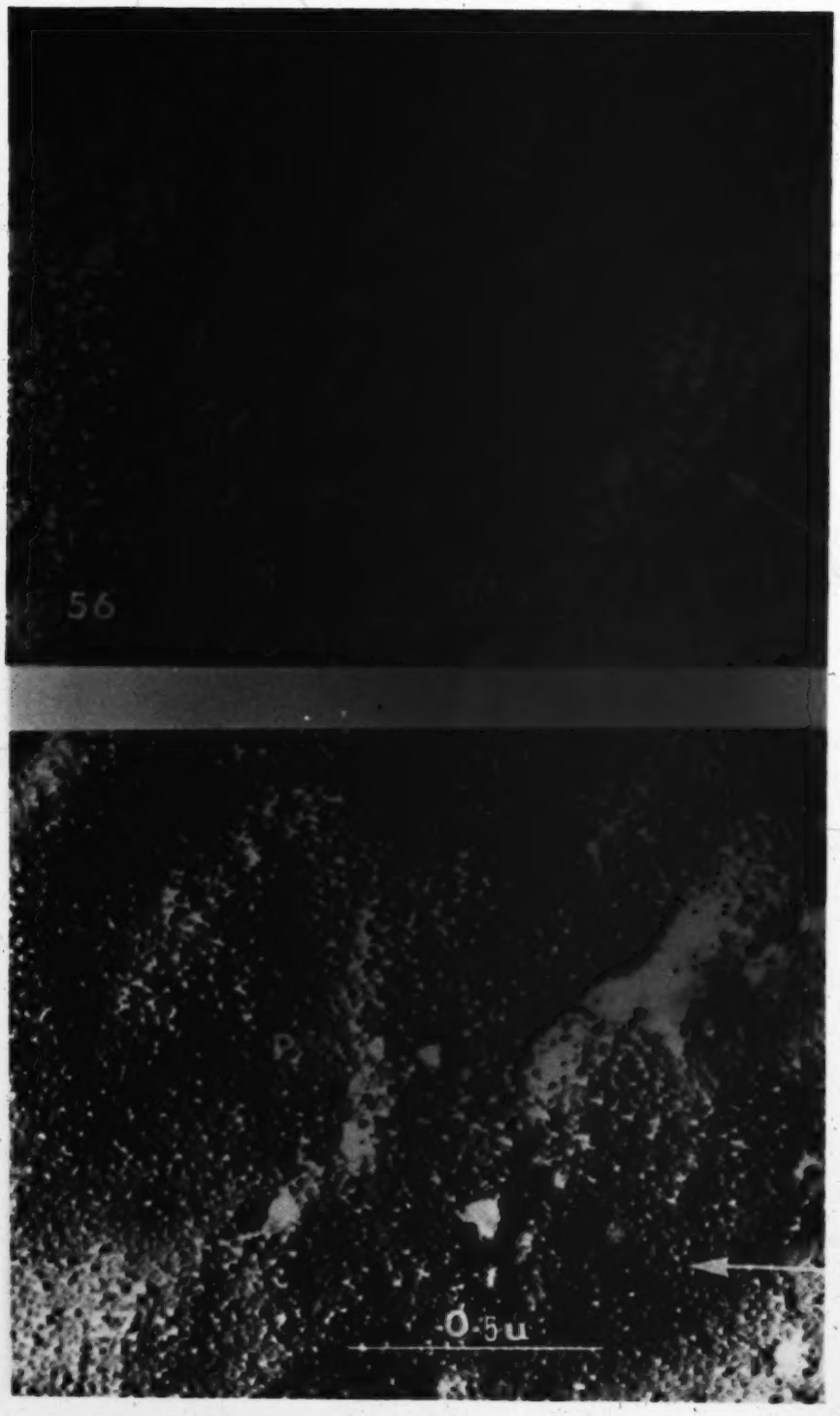

56
P
0·5u

PLATE 39

Figure 58. Fractured faces of synaptic sarcolemma of a muscle denervated for 15 days. There is no change in the distribution or number of particles either on E-face (E) or P-face (P). Compare it to the fractured faces of non-synaptic sarcolemma of control muscle(Fig. 23).

P

PLATE 40

Figure 59. Diagramme summerising the features of control muscle. Basement lamina (BL), pinocytotic vesicles (PV), paired mitochondria (m), A-band (A), I-band (I), M-line (M), Z-band (Z), nucleus (N), nerve terminal (NT), synaptic vesicles (SV), post-synaptic folds (PSF), microfilament (MIF), soleplasm (SP), sarcoplasmic reticulum (SR), transverse tubule (TT), terminalcisternae (TC), peripheral portion of sarcoplasm (PPS), ribosomes (R), endoplasmic reticulum (ER), uniformly distributed AchE activity (AC) , fractured P-face (PS) and E-face (E) of synaptic sarcolemma, P-face (P) and E-face (E) of non-synaptic sarcolemma. my indicates myelinated nerve fiber.

Fig-59

PLATE 41

Figure 60. Diagrammatic summary of the features of denervated m muscle. With the exception of the fractured faces of the synaptic sarcolemma which do not change but are nevertheless labelled only the altered features of the denervated muscle are labelled.

Reduction in the number of synaptic folds (PSF) which show non-uniform distribution of AchE activity (AC). Bundles of microfilaments (MIF), lysosomes (LY),and membrane whorls (MW) are seen in the soleplasm.The abbreviations dF and dZ indicate disrupted filaments and disrupted Z-band respectively. In the longitudinal section, the myofilaments (MF) in transverse morphology can be seen. The illustration shows the notable increase in ribosomes and profiles of endoplasmic reticulum (indicated by R and ER) in the peripheral sarcoplasm (PPS). LI indicates lipid globule. E-face (ES) and P-face (PS) of synaptic sarcolemma do not show any change. PI, PII, PIII indicat indicate the P-face of non-synaptic sarcolemma. E indicates E-face of non-synaptic sarcolemma.

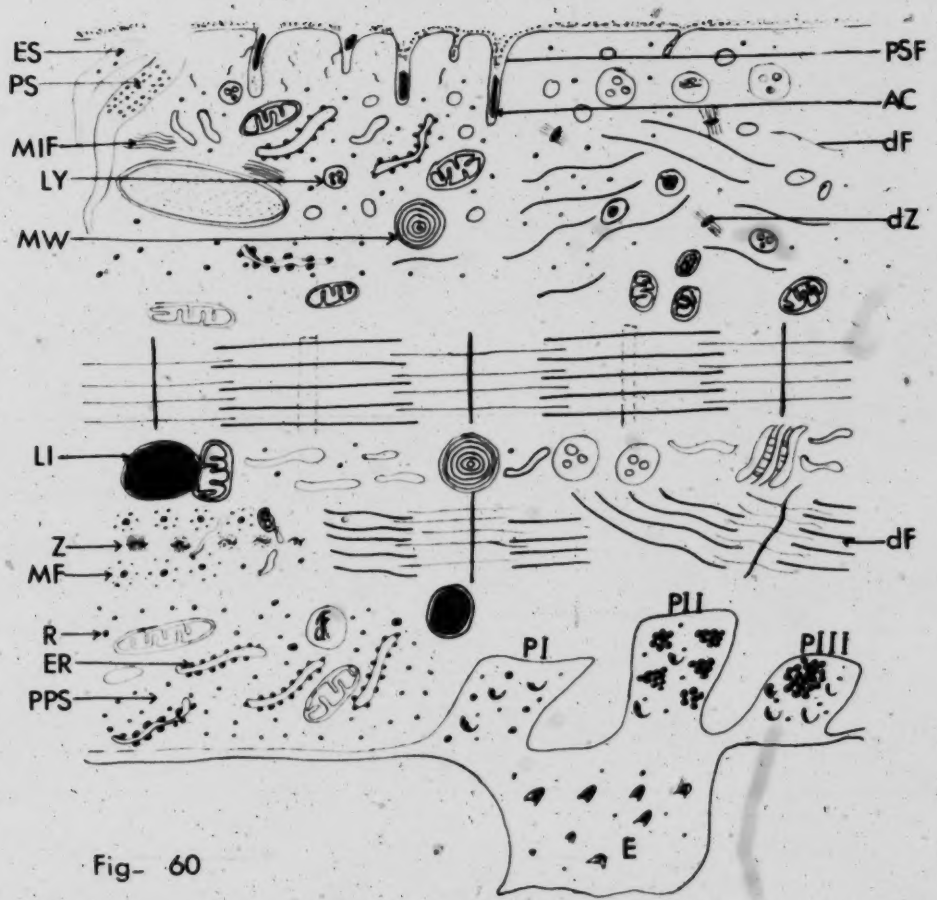


Fig- 60

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